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Quantification of Maternal Offloading of Organic Contaminants in Elasmobranchs Using the Histotrophic Round Stingray (*Urobatis halleri*) as a Model

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Supporting Information

ABSTRACT: Maternal offloading is one route by which young animals may accumulate persistent organic pollutants, such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs), but has not been well documented in elasmobranchs despite their propensity to accumulate high concentrations of contaminants. Using the round stingray (*Urobatis halleri*) as a coastal elasmobranch model, we examined maternal offloading processes at two stages in the stingray's entire reproductive cycle. Post-ovulated and near-term pregnant female stingrays were sampled from southern California, and organic contaminants were measured in the ova and embryonic tissues and compared to concentrations measured in corresponding female livers to determine route and extent of transfer. Total organic contaminant loads measured in ovulated eggs were about two times lower than loads measured in embryos ($p < 0.001$) indicating mothers have the ability to transfer contaminants throughout pregnancy. Contaminant loads measured in pups showed a positive relationship with mother's contaminant concentrations ($p < 0.001$); however, mothers offloaded relatively low percentages (1.5 ± 1.7%) of their total contaminant load using contaminants measured in the liver as a proxy. However, histotrophy is only one form of supplemental provisioning utilized by elasmobranchs and variation in reproductive modes likely influences the extent to which female elasmobranchs may maternally offload contaminants.

INTRODUCTION

Polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT) and its metabolic and environmental breakdown products (DDE and DDD) are particularly problematic contaminants because they are lipophilic, resistant to biodegradation, and biomagnify in the fatty tissues of upper trophic level predators.[1] Besides acquiring contaminants from dietary exposure,[2] some species show differences in contaminant concentrations between age and sex classes, indicating that contaminant accumulation can also be influenced by reproduction. Females, unlike males, have the ability to offload contaminants to their offspring, since they provide a direct energetic contribution to nourish developing young.[3] Although mammalian females have been documented to offload contaminants by two pathways, placental transfer during gestation and lactation,[4,5] a majority of contaminants are transferred to young via lactation. During lactation, organochlorines passively follow lipids that are mobilized from blubber to produce lipid-rich milk, which is subsequently consumed by nursing young.[6,7] Since females transfer a substantial portion of their lipid reserves during lactation, organochlorines are transferred to offspring at a greater rate than during gestation.[4]

Elasmobranchs are another group of animals that invest substantial resources into producing well-developed, precocial young and have the potential to offload contaminants to their young as well.[8] Elasmobranchs have an equivalent energy storage organ to blubber (i.e., large lipid-rich livers) where energy is derived to provision young and is the major site where contaminants can accumulate to high concentrations.[9,10] During egg yolk formation, females transfer hepatic lipids to maturing oocytes via a lipoglycophoshoprotein called vitellogenin.[11] Accumulated contaminants are expected to passively follow hepatic lipids as they are mobilized and redistributed in a process similar to milk formation in marine mammals. In addition to large yolk-filled eggs, many viviparous elasmobranchs provide additional nutrition to embryos in the form of yolksac-placental conveyance, oophagy (ovulation of additional unfertilized ova consumed by embryos *in utero*), uterine secretions (histotroph), and/or intrauterine cannibalism.[8] These supplemental provisioning strategies may represent alternative pathways by which contaminants can be transferred to offspring throughout gestation and influence the extent of maternal offloading across reproductive modes in elasmobranchs.

Due to their high trophic positioning, many marine mammals bioaccumulate considerable contaminant loads. Since they serve as a comparable model for humans most research on maternal transfer of contaminants to offspring has focused on marine mammals. Until recently, studies on maternal offloading

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processes in elasmobranchs have been greatly lacking.[12] The low reproductive output of many elasmobranchs and difficulty in obtaining samples have limited the number of in-depth studies examining maternal offloading process using mother-pup pairs.

The round stingray (*Urobatis halleri*) is an abundant species that forages in close proximity to heavily contaminated sediments in southern California[13] and may represent a suitable model for investigating maternal offloading processes in detail. Round stingrays have one of the shortest gestational periods (approximately 3−4 months) of any elasmobranchs. Since embryos deplete their yolk sacs after the first month of development, mothers provide embryos with supplemental nutrition in the form of histotroph, which nourishes developing young for several months until parturition.[14] Therefore, females have the ability to transfer contaminants to offspring via two routes: ovulated eggs and histotroph. Using the round stingray as an elasmobranch model for species with both a lecithotrophic and histotrophic gestational phase, the objectives of our study were to (1) identify pathways of contaminant transfer from mothers to offspring; (2) determine how factors such as maternal age, contaminant concentration, and fecundity influence the amount females offload; and (3) quantify and compare the proportions of three organic contaminant groups (PCBs, chlordanes, DDTs) transferred from mothers to embryos. Examining maternal offloading processes in detail in a species such as the round stingray may allow us to gain insights and make inferences about similar processes occurring in other, more difficult to study elasmobranchs.

EXPERIMENTAL SECTION

Sample Collection. Stingrays were collected in the summer and fall of 2010 and 2011 corresponding to the events of the stingray's reproductive cycle[14,15] at Seal Beach, Colorado Lagoon, and the Seal Beach National Wildlife Refuge, California (Figure S1). Preovulatory (n = 18) and ovulated females (n = 17) were collected near the size of maturity (15.7−17.6 and 14−17.7 cm disk width [DW], respectively). Pregnant females were also collected based on disk width to obtain a wide age range of mothers (16.0−33.0 cm DW, n = 69). Pregnant females were visually selected based on the degree of abdominal distension,[16] and mid- to late-pregnancy females were sampled.

Animals were collected using a large (26 m long × 3 m tall and a 2 m cod end, mesh size 5 and 1.5 cm) or a small (15.2 m long × 1.8 m tall by 0.32 cm mesh) beach seine net. Upon capture, stingrays were sexed, measured (DW, nearest 0.1 cm), and gestation stage was visually assessed for pregnant females. Stingrays were transported back to California State University, Long Beach, (CSULB) where dissections took place. Stingrays were euthanized by immersion in a seawater ice slurry for 30 min followed by spinal pithing, in accordance with approved CSULB IACUC Protocol # 273. Once rays were euthanized, total body and liver weight were obtained and a piece of the left liver lobe was sampled. Preovulatory ova (herein "ova", no. females n = 18) and ovulated eggs (herein "eggs", no. females n = 17) were dissected from the ovary or uterine horns and weighed to the nearest 0.01 g. Embryos were dissected from pregnant females and sex, disk width, and total body, digestive tract (stomach, spiral valve, spleen, and pancreas), and liver weights (0.01 g) were obtained. Embryos were analyzed as litters by pooling and homogenizing the digestive tract and liver from littermates (no. litters = 69); a pilot study previously demonstrated negligible amounts of contaminants in non-visceral tissues (Figure S2). Therefore, all subsequent results for contaminants measured in embryos herein refer to those derived from embryonic visceral tissues (i.e., liver and digestive tract). However, embryos near parturition size from one litter were analyzed as whole individuals to test our assumption that contaminants are distributed equally among littermates. All tissues used for organic contaminant (OC) analysis were subsequently wrapped in foil and stored at −20 °C until chemical analyses could take place.

Chemical Analyses. Tissue extractions and contaminant quantifications were performed at CSULB's Institute for Integrated Research on Materials, Environment and Society. Each sample extract was analyzed for DDT and its derivatives (n = 6), chlordanes (oxychlordane, gamma-, alpha-, trans-, cis-chlordane), and 54 congeners of PCBs and summed to obtain total DDT ("DDTs"), chlordanes ("CHLs"), and PCBs.

Following previously described methods,[18] homogenized ova and embryonic tissues and subsamples of female livers were extracted for 14−16 h via a Soxhlet apparatus in 100% methylene chloride (DCM). Prior to extraction, all samples were spiked with a known quantity of recovery surrogates (TCMX, PCB 30, 112, and 198) to measure efficiency of preparative and analytical procedures (target recovery of 70−130%). Sodium sulfate was added to embryo samples due to their relatively high water content. After extraction, samples were concentrated by rotovap and lipid content was determined gravimetrically from split aliquots. Extracts were then purified through elution through an Alumina-B/Silica gel with hexane, 30% DCM in n-hexane, and DCM and concentrated. Due to small sample weights, ova extracts were transferred to autosampler vial inserts and concentrated (≤100 μL) to increase detection resolution. 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 (MSD; Agilent 5973 inert series). The GC column employed was a ZB-5 (Phenomenex; Torrance, California) fused silica capillary (0.25 mm ID × 60 m) with 0.25 μm film thickness. The temperature profile of the GC oven was programmed from 45 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 and 300 °C, respectively. The source and quadrupole temperatures were set at 230 and 150 °C, respectively. Helium was used as the carrier gas at a flow velocity of 40 cm/s. The MSD was operated in the electron ionization (EI) mode and scanned from 45 to 500 amu at a rate of 1.66 scans/s. Concentrations of organic contaminants were quantified using the software in the GCMS system (Agilent Technologies).

Quality Assurance/Quality Control. Quality assurance quality control samples were run in tandem with each batch (n = 12) of study samples to ensure accuracy and precision of data acquired and included one blank, one study sample replicate, two duplicate matrix spikes, and one 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 QC goal was for 90% of the replicates to yield a relative percent difference (RPD) of <30% with recovery of spiked analytes at 70−130%.

The mean ± SD of recovery surrogates was 120 ± 29%, 111 ± 24%, 125 ± 25%, and 84 ± 23% for TCMX, PCB 30, 112,

and 198, respectively, which demonstrated acceptable efficiency of procedures. Recovery of CRM analytes among batches was 94 ± 8% for PCBs and 90 ± 11% for pesticides and blanks showed no signs of procedure contamination. Mean relative significant differences between replicates of sample duplicates and matrix spikes were relatively low (13 ± 14% and 8 ± 9%). Mean recovery of matrix spikes was 91 ± 6% and 82 ± 10% for PCBs and pesticides. Therefore, QA/QC samples satisfied criteria and data were not corrected for recovery.

Data Analysis. OCs per sample were summed as a whole (herein "summed OCs") and reported as either concentration (wet [ww] or lipid [lw] weight basis) or total load (ng). Total load was calculated by multiplying ww concentration by the total weight of the organ or tissue analyzed. OCs for ova, eggs, and embryos were reported as "standardized total load" (i.e., OCs per number of ova, eggs, or embryos obtained from each female [ng/#]) since tissues were of small enough weight to be analyzed whole. Where percentages were compared, values were arcsin transformed prior to analysis.

Ova and Eggs. Factors that were thought to influence contaminants measured in ova and egg tissues were their weight, females' liver concentration, and female's disk width. Therefore, natural log (LN) transformed values were used in a multiple regression to determine the relationship between these factors and measured contaminant loads in ova and eggs. In addition, the percent of a female's total contaminant load that was transferred to ova or eggs (herein "percent offloaded") was compared by t test. A pilot study comparing organic contaminants measured in stingray liver and extra-hepatic tissues (i.e., whole rays excluding liver, $n = 7$) demonstrated that organic contaminant load ([OC] × total tissue weight) found in nonliver tissue contributed very little (3.3 ± 1.6%) to the total body load (Figure S3). Therefore, contaminants measured in livers were used as a proxy for total contaminant load of the animal. The offloading percentages were calculated by the following formula: (egg or ova load)/(female total liver load + egg/ova load) × 100, assuming the contaminant concentrations were homogeneous throughout her liver. Females were expected to have offloaded more contaminants to eggs (fully developed ova) compared to nearly developed (preovulatory) ova found in the ovary.

Eggs and Embryos. Developing embryos typically deplete their yolk reserves by the end of the first or second month at which time females will secrete histotroph to nourish embryos until parturition. Since females provide their young with supplemental nutrition, they have the opportunity to continually offload contaminants throughout pregnancy. To test this hypothesis, we first compared the LN transformed standardized loads offloaded between eggs ($n = 17$) and a subset of near-term embryos ($n = 10$) using Welch's t tests from females of comparable disk widths (15.7−17.6 and 16−17.8 cm DW, respectively) so that females were of similar ages. To ensure that any differences found between eggs and embryos were not due to differences in female contaminant loads before reproduction, female loads prior to ovulation were back calculated by adding egg or embryo loads to female total loads and comparing LN transformed values through a t test.

In addition to total amount of contaminants offloaded, we were also interested in comparing the types of contaminants that were transferred during different stages of reproduction. The percent of ΣPCBs, ΣDDT, and Σchlordanes measured per sample were compared between eggs and embryonic tissues through a generalized linear model using a beta distribution with a logit linked function in SAS 9.3. PCBs were further subdivided into groups by number of chlorinated congeners (i.e., tri, tetra, penta, hexa, hepta, octa, nona) and the proportions compared between embryos and eggs. PCB 209 (deca congener group) was removed from analysis due to number of samples where PCB 209 was detected. Proportions were calculated by dividing the sum of each chlorinated congener group by the total amount of PCBs measured per sample.

Mothers and Embryos. Female age (i.e., disk width) and contaminant concentration were hypothesized to influence the amount of contaminants offloaded. In other species, older females have been shown to offload significantly fewer contaminants to their offspring compared to younger females[17] and we expected to see a similar pattern. In addition, the amount of contaminants a female acquires prior to a reproductive event might also play a role in the amount she may transfer to young, where females with higher loads may transfer more to their offspring.[18] We explored these relationships by performing a multiple regression using the unstandardized and standardized total loads measured in a litter against female's disk width, liver concentrations, and total liver load. No relationship was found between their liver lipid content and size ($p = 0.57$) or correlation of contaminant concentration with lipid content ($p = 0.25$); therefore, wet weight concentrations were used. However, female's liver weight did increase with size ($F_{1,67} = 266$, $p < 0.0001$, $R^2 = 0.80$). Normalization of the data to mother's body mass was explored but did not alter the observed patterns; outcomes of this analysis were not included in the results.

Since larger females tend to produce larger litters, we might expect offloaded contaminants to show a "dilution effect" since contaminants can be distributed among more offspring. Therefore, we examined the relationship between standardized LN total litter load and number of embryos per litter through linear regression. In addition, if females offload contaminants continuously throughout gestation we expected the amount of contaminants per litter to increase with increasing disk width of embryos. However, since litter load may be related to their mother's concentrations, we first normalized the standardized litter load to mother's total load.

Lastly, we were interested in the types and proportions of contaminants that females transferred to their offspring. Proportions of offloaded PCBs, chlordanes, and DDTs were calculated by dividing the embryo load of each contaminant group by the summed total load (mother and embryo). Offloaded arcsine transformed proportions were then compared with an ANOVA followed by Tukey's posthoc test. A similar GLM as described above was used, except a repeated measures function was included to account for mother-pup pairs to compare the proportions of PCB congener groups.

RESULTS

Ova and Eggs. Summed OC loads were significantly higher in eggs (132.6 ± 58.2 ng/egg) than in preovulatory ova (71.63 ± 47.7 ng/ova; $t_{33} = 4.22$, $p < 0.001$; Figure 1). Likewise, the percent of offloaded contaminants was approximately twice as high in eggs compared to ova (0.51 ± 0.23% versus 0.28 ± 0.24%); however, in the multiple regression of LN summed OC load measured in egg and ova tissues significantly increased with their weight ($F_{3,27} = 23.7$, $p < 0.001$, $R^2 = 0.69$). While summed OC load in ova and eggs increased with weight, the proportion of PCBs and pesticide contaminants measured in

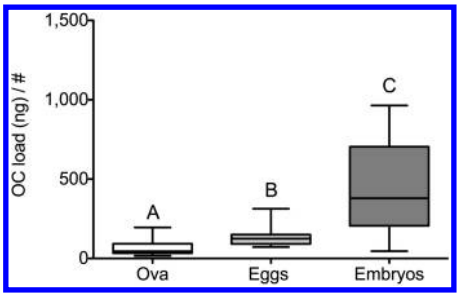

Figure 1. Mean ± SD of summed organic contaminant (OC) load per litter divided by the number of embryos in each litter (no. of litters = 10) were significantly higher (*t* test, $p = 0.0006$) than summed OC load measured per egg (no. ovulated females = 17), and OC load per egg was significantly (*t* test, $p < 0.001$) greater than those per ova (no. preovulatory females = 18). Female stingrays from which these tissues were taken had comparable hepatic OC concentrations ($p = 0.91$). Whiskers represent min and max values and different letters represent significant differences.

these tissues did not change with size increase ($F_{1,33} = 0.68$, $p = 0.41$; Table S1). Although eggs were significantly heavier in weight than ova ($t_{31.5} = 3.47$, $p = 0.002$), the percent lipid content was comparable between these two tissues ($t_{19.8} = -0.03$, $p = 0.97$) and there was no relationship between lipid content and measured contaminants ($F_{1,23} = 0.005$, $p = 0.94$). Furthermore, when compared on a lipid weight basis, ova and egg contaminant concentrations were no longer different with the removal of one ova outlier ($p = 0.2$; Table 1). Of the two female related factors used in the multiple regression, only female's contaminant concentrations ($p = 0.025$) demonstrated a significant relationship with the LN OC load in eggs and ova, and no relationship was found with disk width ($p = 0.9$).

Eggs and Embryos. Mean ± SD of summed OC loads measured in eggs were significantly lower than those found in embryos from females of comparable sizes (438.66 ± 301.64 ng/embryo; $t_{25} = 3.9$, $p = 0.0006$; Figure 1). Similarly, the mean percent of offloaded contaminants was significantly greater in late-pregnancy than ovulatory females (1.83 ± 1.58 and 0.52 ± 0.23%; $t_{25} = -4.4$, $p = 0.0002$). While the estimated contaminant load of these females prior to this reproductive event was not different ($p = 0.91$), we did observe a significant decrease in liver lipid content between ovulating and late-pregnancy females ($t_{25} = 2.6$, $p = 0.012$).

Proportions of PCBs, DDTs, and chlordanes were not significantly different between eggs and embryos ($F_{2,67} = 2.36$, $p = 0.10$); however, within these groups proportions by contaminant type differed depending on the number of embryos or eggs sampled per female ($p = 0.008$). As number of embryos or eggs in a "litter" increased, the proportion of PCBs decreased ($p = 0.04$) while the proportion of DDT increased ($p = 0.02$). The mean ± SD of summed OC load of PCBs (636.9 ± 507.9 and 169.1 ± 77.9 ng, respectively), DDTs (46.38 ± 14 and 37 ± 9.2 ng, respectively), and chlordanes (138.4 ± 139.0 and 31.8 ± 13.0 ng, respectively) were significantly higher in embryos than eggs ($p < 0.001$). When PCB proportions were separated by chlorinated congener group, embryos and ova were found to have significantly different proportions for three out of the seven groups ($p < 0.001$, Figure 2A). Eggs had higher proportions of the most chlorinated congeners (nona $p = 0.003$) and least chlorinated congeners (tri, $p < 0.001$). Deca congeners were only measured in egg tissues. Embryos had higher proportions of the less chlorinated congeners (i.e., tetra and penta, $p = 0.06$ and 0.004). Eggs and embryos were similar in proportion for hexa, hepta, and octa congener groups ($p = 0.9$, 0.7, and 0.2).

Mothers and Embryos. While the average percent of offloaded contaminants was relatively low (1.5 ± 1.7%) it was highly variable and showed a decreasing relationship with female size ($F_{1,67} = 6.0$, $p = 0.016$). No relationship was found between average embryo disk width and their mother's liver weight normalized to her disk width ($p = 0.24$). However, the standardized (i.e., per embryo) and unstandardized litter LN contaminant loads showed a positive relationship with their mother's liver contaminant concentration ($F_{3,64} = 10.73$ and

Table 1. Organic Contaminants Measured in the Livers of Pre-Ovulatory and Ovulating Females and Their Ova (Outlier Removed, $n = 17$) and Eggs and Those Found in Embryos and Their Mother's Liver Are Reported as Total Load, Wet Weight Concentration (ww), and Lipid Weight Concentration (lw)[a]

sample	total load (μg/# or μg)	*n*	% lipid	[ww] (μg/g)	[lw] (μg/g)
Ova	0.07 ± 0.04		5.1 ± 1.9	0.17 ± 0.1	2.9 ± 1.0
Females	27.5 ± 13.9	18	47.8 ± 11	1.4 ± 0.64	3.0 ± 1.4
Eggs	0.13 ± 0.06		9.0 ± 0.59	0.22 ± 0.11	2.5 ± 1.1
Females	43.7 ± 12.3	17	58.0 ± 6.8	1.7 ± 0.5	3.0 ± 1.1
Embryos					
5.0−5.5	0.17 ± 0.14		2.4 ± 3.6	0.21 ± 0.14	8.3 ± 5.6
Mothers	66.9 ± 36.1	12	47.6 ± 8.5	2.4 ± 0.1.2	4.5 ± 2.2
5.51−6.0	0.32 ± 0.2		2.8 ± 0.85	0.21 ± 0.08	11.6 ± 8.1
Mothers	44.5 ± 7.4	11	44.5 ± 7.4	2.7 ± 0.9	4.7 ± 1.5
6.01−6.5	0.44 ± 0.31		1.8 ± 1.0	0.17 ± 0.09	9.3 ± 7.6
Mothers	132 ± 85	19	45.6 ± 9.0	3.0 ± 1.5	4.8 ± 2.5
6.51−7.0	0.40 ± 0.21		2.5 ± 1.7	0.15 ± 0.07	10 ± 6.2
Mothers	82.9 ± 44.5	12	47.7 ± 11.6	2.6 ± 0.82	3.9 ± 1.3
7.01−7.5	0.80 ± 0.36		3.0 ± 2.4	0.17 ± 0.13	9.6 ± 9.4
Mothers	178 ± 192	7	37.9 ± 20.5	3.9 ± 1.9	5.4 ± 2.6
7.51−8.12	0.68 ± 0.47		1.6 ± 0.7	0.16 ± 0.11	6.3 ± 3.4
Mothers	94.9 ± 53.4	6	48.0 ± 4.3	2.1 ± 0.51	2.7 ± 0.6

[a]Total load represents the product of wet weight (ww) concentration found in the sample multiplied by the weight (g) of tissue analyzed. Ova, eggs, and embryo total loads were standardized to the number sampled from each female or mother (i.e., μg/#).

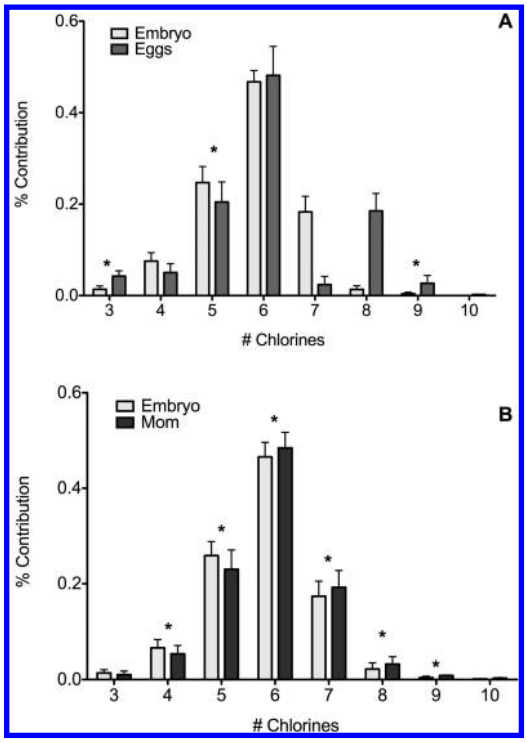

Figure 2. (A) Proportions PCB congener groups in embryos (light gray bars) were similar to those in ovulated eggs (gray bars), except for tri, penta, and nona congener groups. (B) Embryos had significantly higher proportions of tetra and penta PCB congeners compared to their mother's liver (dark gray bars) that had higher proportions of heavier chlorinated PCB congener groups (hexa-nona). Asterisks denote significant differences between embryos and ovulated eggs or mothers.

17.06, $p < 0.0001$, $R^2 = 0.30$ and 0.42; Figure 3). Mother's total load was not significant ($p = 0.12$) and size only showed a

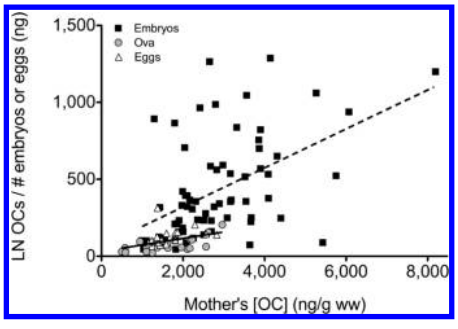

Figure 3. Natural log (LN) of summed OCs (ng) measured per litter per embryo (black triangles) and per ova (gray circles) and egg (gray triangles) showed a significant, positive relationship with increases in their mother's hepatic OC concentration [ww] ($n = 69$, $p < 0.0001$, $R^2 = 0.32$ and $n = 35$, $p = 0.009$, $R^2 = 0.16$, respectively). However, the rate of increase was significantly greater in the embryos (dashed line) than the ova/eggs (solid line; ANCOVA $F_{1,100} = 20.4$, $p < 0.0001$).

positive relationship with litter contaminant loads when they were not standardized per embryo ($p = 0.01$).

While a significant relationship was found between the LN of the total unstandardized litter load and number of embryos in a litter ($F_{1,67} = 5.6$, $p = 0.02$), standardized OC load with respect to litter size was marginally insignificant ($F_{1,67} = 3.5$, $p = 0.06$). Contaminant loads in one litter of near-term embryos (PF-14, $n = 5$) were analyzed individually and all embryos except for one, whose lipid levels were lower than his littermates, showed very little difference in contaminant load (1063 ± 117 versus 603 ng summed OCs; Figure S4). Contaminants measured in litters were also influenced by the stage of gestational development. The LN of standardized embryo load, which were normalized by their mother's liver concentration (i.e., ng/embryo/[mother's OC]), increased as the average disk width for the litter increased ($F_{1,67} = 29.0$, $p < 0.0001$, $R^2 = 0.21$). A nonlinear regression of standardized litter load (normalized to their mother's liver concentration) with embryo size showed a similar pattern to that of the relationship between average litter weight and disk width ($R^2 = 0.38$ and 0.93, respectively; Figure 4). In addition, concentration by lipid weight becomes substantially greater than their mother's as the size class of embryos increased (Table 1).

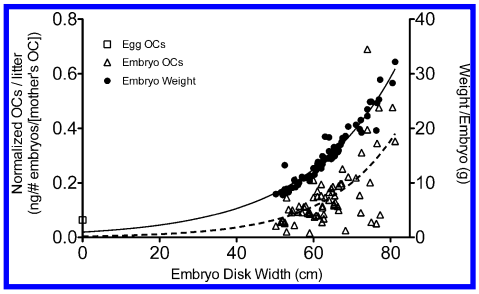

Figure 4. OCs measured per litter per embryo normalized to their mother's liver OC wet weight concentration (open triangles) increased in a nonlinear fashion as the average litter disk width increased (dashed line). In addition, OCs measured in embryos were substantially greater than the average OCs found in eggs (open square). Increases in litter OCs with disk width was similar to the relationship found between average litter weight per embryo and disk width (closed circles, solid line) during the mid to late gestational (no. of litters = 69).

Embryos showed similar contaminant profiles as their mothers in that mean ± SD load of PCBs comprised a majority of the total contaminant load (1062 ± 1012 ng; 81.4 ± 6.2%) with chlordanes representing the next largest contaminant group (194.0 ± 230.9 ng; 14.3 ± 6.9%) followed by DDT (51.2 ± 48.3 ng; 4.2 ± 2.2%; Table S1). However, the LN of the offloaded proportion of these three contaminant groups was significantly different (ANOVA $F_{2,202} = 7.34$, $p = 0.0008$). DDT was found to be offloaded at a higher proportion (2.66 ± 2.68%) compared to PCBs and chlordanes ($p = 0.002$ and 0.003, respectively), while PCBs and chlordanes were comparable (1.46 ± 1.6% and 1.63 ± 2.62%; $p = 0.98$; Figure 5). The PCB chlorinated congener groups also showed similar patterns between embryos and mothers, with hexa congeners making up the largest proportion of PCB contaminants when pooled (47.4 ± 3.2%), followed by penta (24.4 ± 3.8%), hepta (18.3 ± 3.5%), tetra (5.9 ± 1.8%), octa (2.7 ± 1.5%), tri (1.3 ± 0.7%), nona (0.64 ± 0.42%), and deca (0.2 ± 0.1%) congener groups. However, the relative proportions of each congener group were different between mothers and embryos for all congener groups ($p < 0.009$) except for tri congeners, which were marginally insignificant ($p = 0.051$, Figure 2B). Embryos were found to have higher proportions of tetra and penta congener groups, while mothers had higher proportions of the more chlorinated groups (i.e., hexa-nona).

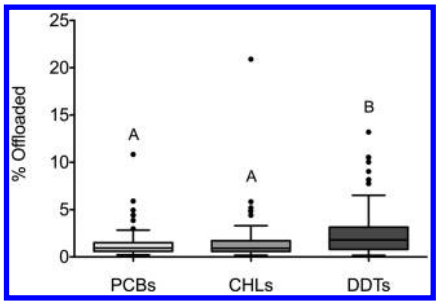

Figure 5. Proportion of offloaded contaminants measured in embryos (n = 69) was significantly different (p = 0.008) among groups with DDT (dark gray) having higher rates than PCBs (light gray) or chlordanes (gray). Boxes represent the first and third quartile and dark lines indicate group means.

DISCUSSION

Female round stingrays were found to maternally offload contaminants to their offspring via two pathways. One route of transfer was through the production of yolky eggs, which embryos utilized during the first third of the gestation period.[14] During the energetic process of vitellogenesis, females redistribute lipids from their livers to ova.[11] Livers are the main energy storage organ in elasmobranchs and thus tend to have the highest contaminant concentrations.[19,20] Therefore, as hepatic lipids are mobilized and transferred to ova contaminants will passively follow. The positive relationship found between ova weight and contaminant load suggests that females continually transfer contaminants to ova throughout their development until ovulation. Given the comparability of ova and egg concentrations on a lipid weight basis suggests that lipid transfer is the vehicle by which contaminants are transferred to these tissues. In addition, females with higher contaminant concentrations transferred higher loads to eggs and ova. Therefore, female's contaminant concentration seems to be an important factor influencing the total amount of contaminants that can be transferred to offspring. Ovulated eggs had significantly higher contaminant loads compared to near ovulation sized ova. Although the percent lipid content did not differ between the two, the larger weight, and therefore total lipid content of ovulated eggs, likely results in greater transfer of these lipophilic contaminants compared to developing ova, which have not yet reached complete maturation.

The second route by which female round stingrays were shown to offload contaminants was through the production of histotroph, or uterine milk,[14] which embryos consumed as a supplemental form of nutrition for a majority of gestation until parturition. The significantly higher loads of summed OCs and greater offloading percent of near-term embryos compared to ovulated eggs demonstrates that females are able to continually transfer contaminants to offspring during gestation. Females of comparable sizes were chosen to remove any potential age influences, since older females would have more time to accumulate contaminants than younger females, which was important to consider since maternal hepatic contaminant concentrations were found to significantly influence the amount of contaminants females offloaded to both eggs and embryos. Assuming homogeneous liver contaminant concentrations, pre-reproductive loads calculated for ovulated and pregnant females were not significantly different in their total contaminant load. Therefore, the higher loads in embryos compared to eggs is due to additional transfer during gestation rather than prior differences in female's contaminant load or size.

While the exact lipid content may vary among species, histotroph is rich in lipids[21] and could result in substantial contaminant transfer. Fatty acids of histotroph measured in two species of rays (butterfly ray, *Gymnura micrura*, and cownose ray, *Rhinoptera bonasus*)[22] were found to be very similar in their composition to those measured in human (*Homo sapiens*) and bovine (*Bos taurus*) milk. Unfortunately, we were unable to measure the contaminant concentrations of histotroph due to uterine flushing by females, which would likely result in lower than actual measured loads. However, late pregnancy females had significantly lower concentrations of hepatic lipids than ovulating females. We assume this decrease in lipid results from continued energetic input, and therefore contaminant transfer, to offspring during gestation, which has been documented in other batoid rays.[23] Since round stingrays undergo continual oogenesis and vitellogenesis it is likely these processes will contribute to the decrease in maternal hepatic lipids as well. However, since developing oocytes in sampled females were small in size (~0.1−0.4 cm diameter), number (n = 1−3), and still at an early developmental stage[14] (K. Lyons, personal observation) the proportion of lipids directed to oocytes versus embryos is expected to be small.

In addition, standardized embryo contaminant load increased in a similar pattern to embryo growth rate as development progressed. This further supports our hypothesis that females transfer contaminants to offspring during gestation at least for the midlate developmental stages. If embryos did not continually accumulate contaminants during development, contaminant loads would decrease as embryos reached parturition size, which was not observed. Furthermore, embryo lipid weight contaminant concentrations were consistently greater than their mother's liver concentrations, which corresponds to similar observations made in marine mammal systems.[7,27] This highlights the transfer of organic contaminants via lipids from mothers to embryos and their subsequent concentration in neonatal tissues. This supplemental provisioning by mothers is important not only for continued embryo growth throughout gestation, but also for the accumulation of energy reserves that offspring will depend on postpartum until they can competently feed on their own.[24] Indeed, embryos further in development had relatively larger livers compared to the weight of other visceral organs (i.e., stomach and spiral valve) than embryos that were less well developed (K. Lyons, unpublished data).

While standardized loads were higher in embryos, the contaminant proportions of PCBs, DDTs, and chlordanes were comparable between eggs and embryos. Therefore, females probably do not transfer these three contaminant groups at different rates during egg formation or throughout pregnancy. Although total PCB proportion was similar, the composition of PCBs by chlorinated congener group was significantly different between eggs and embryos, indicating differential transfer rate at these two points in reproduction (i.e., vitellogenesis and pregnancy). Less chlorinated PCB congeners tend to be more labile and less lipophilic than heavier, more chlorinated congeners.[25] Therefore, the lipophilicity of different PCB congeners will influence their mobility and thus ability to be transferred. Congener groups that had fewer chlorines (tetra and penta) were found in higher proportions in embryos than in eggs. Although very low in proportion and load, the most chlorinated congeners (deca) were only measured in eggs.

Indeed, maternal offloading studies in marine mammals have demonstrated that PCB congeners are transferred at differential rates, with the lighter congeners being transferred more easily.[26−28] In addition, PCB transfer may also be influenced by their affinity for different types of lipids,[29] which may be mobilized at various stages of reproduction.[7] In the round stingray, the types of lipids used for yolk formation may differ between those utilized for histotroph secretion, which could lead to differences in the proportions of contaminants transferred if lipids vary in their hydrophobicity. Since higher proportions of the more chlorinated congeners were found in eggs compared to embryos, this suggests that more nonpolar lipids may be transferred to eggs than during the histotroph phase of gestation, but this remains to be explored.

Maternal hepatic contaminant concentrations appeared to be the most influential factor accounting for contaminant load offloaded to eggs and embryos, regardless if it was standardized by litter size. We may infer that a maternal condition may play an important role in maternal offloading. If females were in a starved or catabolic state, then contaminants would become more concentrated in hepatic tissues as energy stores were utilized. Alternatively, maternal feeding rate and location may influence their contaminant uptake rate, which could lead to higher concentration if it exceeded liver growth rate. In either scenario, subsequent lipid mobilization for reproduction would lead to greater maternal transfer as the amount of contaminants dissolved in those lipids would be higher. In addition, when mothers' liver OC concentrations were normalized to their disk width and liver weight (i.e., [OC]/liver weight/disk width) and compared to the average embryo disk width of the litter a positive relationship was found such that mothers' normalized OC concentrations significantly increased as embryos increased in size during development.

Using disk width as a proxy, age in this study was found to be significant only when unstandardized embryo litter load was used. The explanatory power of age (i.e., disk width) with respect to maternal offloading in this species maybe complicated by the fact that liver growth rate exhibits a linear relationship with disk width (K. Lyons, unpublished data). A contaminant uptake rate that is more or less equal to growth rate may result in rather stable contaminant concentrations despite growth, uncoupling these two variables.

Regardless, the proportion of females' total contaminant load as estimated by the liver that was transferred to offspring was much lower than expected (1.5 ± 1.7%). Since mothers are not fasting during pregnancy, their continued acquisition of dietary contaminants may result in an underestimation of the extent of maternal transfer, to a degree. Contrary to expectation, we found that mother's hepatic OC concentrations increased from the mid to late gestational stages despite the lack of change in female liver weight, which suggests that mother's intake of newly acquired contaminants during gestation was greater than the amount they were offloading. Nevertheless, the results of our study are in stark contrast to maternal offloading studies in other species of elasmobranchs such as white (*Carcharodon carcharias*) and thresher (*Alopias vulpinus*) sharks, which suggest that females transfer a substantial portion of their contaminants to offspring.[12] White and thresher sharks utilize oophagy (where embryos consume unfertilized ovulated eggs throughout gestation[30]), have substantially longer gestational periods, and produce highly developed young.[31,32] Despite differences in supplemental provisioning, round stingrays also have a substantially shorter gestation period and produce young comparatively smaller in size, which would greatly limit the opportunity for females to offload contaminants compared to white or thresher sharks. Given that elasmobranchs demonstrate a wide range of reproductive modes from lecithotrophy to pseudoplacental matrotrophy, varying degrees of maternal investment is likely an important factor influencing the magnitude of maternal transfer in elasmobranchs.

While round stingray females were able to offload more contaminants to larger litters, the amount offloaded per pup in each litter was not related to their mother's size (i.e., age) or the number of siblings in a litter. Since fecundity increases with size in round stingrays as it does in many other species of elasmobranchs, we originally expected embryos from larger females to have fewer contaminants due to (1) hypothesized significant decreases in maternal contaminant concentrations after successive reproductive cycles and (2) a dilution effect due increased number of offspring with concurrent increases in maternal size. The weak relationship between female's size (i.e., age) and hepatic contaminant concentration, which was the most influential factor, was likely the reason the amount of contaminants offloaded per embryo remained relatively constant despite larger litters and older ages in larger sized females. If round stingray females were removing a substantial portion of their contaminants through reproduction, we would expect to see contaminant load per embryo per litter decrease, or become diluted, with increase in litter size, since larger, older females are more fecund, which was not the case.

Although mothers and their embryos showed similar contaminant composition patterns for the three contaminant groups with PCBs comprising a majority, the offloading rates of the three contaminant groups were significantly different. While DDTs made up the smallest portion of the total contaminant load, this contaminant group was offloaded in the highest proportion compared to PCBs and chlordanes. Similar offloading patterns have been observed in many marine mammals species where DDTs are transferred at higher proportions than PCBs[27] due to differences in chlorination, which is related to lipophilicity. The major metabolite of DDT, 4,4′-DDE, which has 4 chlorines, comprised a majority (88 ± 18%, n = 238) of the DDT-related compounds measured. In addition, a large portion of the PCB congeners detected had 6 or more chlorines (70 ± 6%, n = 238). Therefore, the fewer number of chlorines found on 4,4′-DDE compared to PCBs and chlordanes (8−9 chlorines) could make it more easily transferrable and could account for the higher transfer proportion of DDT compared to the other two groups.

The patterns of PCB congener composition found in female and embryo stingrays were similar to those found in other marine organisms such as bottlenose dolphins (*Tursiops truncatus*) from the Gulf of Mexico[26] and ringed seals (*Phoca hispida*) from Canada,[33] highlighting the ubiquity of PCBs despite large geographic separation. However, significant differences between embryos and mothers were found for all PCB congener group proportions, except for tetra congeners. Embryos had higher proportions of tri, tetra, and penta congeners compared to mothers that had higher proportions of the more chlorinated congeners (hexa-deca). These results parallel those found in marine mammal maternal offloading studies.[5,6,26]

Despite the overall low offloading rate of female round stingrays, the loads measured in embryos were substantial and embryos within a litter appear to receive similar amounts of contaminants. While we did not measure any metrics that

might be indicative of negative physiological effects, populations of stingrays in southern California are quite healthy despite the fact that embryos are exposed to potentially harmful chemicals during development and adult females accumulate contaminant loads comparable to higher trophic level elasmobranchs.[34−36] Further studies should continue to explore the dynamic between maternal offloading of contaminants and reproductive mode in elasmobranchs as well as the effect of embryonic and neonatal exposure.

■ ASSOCIATED CONTENT

Supporting Information

Additional data on contaminants and collection sites. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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