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Steroid concentrations in maternal serum and uterine histotroph in round stingrays (*Urobatis halleri*)

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A B S T R A C T

Despite a wide range of elasmobranch (sharks, skates and rays) matrotrophic strategies, and thus potentially diverse pathways for maternal-fetal hormone exchange, little attention has been given to uterine steroids during development. Round Stingrays (*Urobatis halleri*) with matrotrophic histotrophy were captured during every month of their annual reproductive season from post-ovulation to near parturition, and paired samples of plasma and histotroph were analyzed for a suite of steroid hormones using LC-ESI/MRM. Hormone concentrations within and between maternal and uterine compartments were compared using two markers of embryo development. Histotroph had consistently higher detection rates and concentrations of hormones than maternal plasma, especially during early pregnancy when embryos are yolk sac-dependent for nutrition. Peaks in histotroph testosterone concentrations preceded maternal plasma, suggesting that hormones were locally produced within the uterine compartment. Embryonic sexual differentiation based on the presence of visible claspers (male copulatory organs) coincided with peaks in histotroph progesterone, testosterone, 17-hydroxyprogesterone and estradiol, suggesting that, like mammalian pregnancy, elasmobranch embryonic steroids also contribute to their own developmental environment.

1. Introduction

Many elasmobranch fishes (i.e. sharks, skates and rays) are viviparous, exhibiting a wide variety of matrotrophic provisioning strategies ranging from pseudoplacental viviparity to oophagy to histotrophy, with different levels of physiological connectivity between mother and embryos (Wourms et al., 1988; Hamlett et al., 1993; Musick and Ellis, 2005). Evidence for lifelong impacts for developmental stressors, mediated by exposure to maternal steroids, is accumulating in terrestrial vertebrates from birds to reptiles (Meylan and Clobert, 2005; Groothuis and Schwabl, 2008), but there is gap in our understanding of the developmental steroid environment in elasmobranchs.

In mammals, maternal recognition of pregnancy is important to prevent spontaneous abortion and establish an appropriate environment for developing embryos. Steroid hormones play key roles in initiating these changes by quieting uterine contractions and altering uterine secretions. In swine and lagomorphs, blastocysts are steroidogenic as early as 14 and 17 days post-fertilization, respectively (Bazer et al., 1979; Wilson et al., 1980). These embryo-produced signals alter the secretory functions of the uterus related to glucose and protein transport as well as triglyceride synthesis (Forde et al., 2009). In sharks and rays, where maternal investment in pregnancy can be high as it is in mammals, both maternally-derived and embryo-derived steroid hormones might also be important for reproductive success.

Round Stingrays (*Urobatis halleri*) have several characteristics that make them an ideal elasmobranch model. They are abundant, easy to capture and have a well-described annual reproductive cycle (Babel, 1967; Hoisington and Lowe, 2005; Mull et al., 2010). In addition, Round Stingrays employ histotrophic matrotrophy to provide supplemental nutrition to embryos, whereby the uterus produces protein and lipid-rich secretions that nourish embryos throughout development, particularly after they have exhausted their yolk sacs (Babel, 1967; Spieler et al., 2013). Female Round Stingrays will carry embryos in both left and right uteri and undergo one pregnancy per year (Mull et al., 2010). Litter size ranges from 1 to 8 embryos, and linearly increases as females grow (Lyons and Wynne-Edwards, 2018a). From the beginning of pregnancy, both the left and right uteri are densely lined with highly vascularized uterine villi (trophonemata) that transfer nutrients from the maternal compartment to the embryonic compartment through uterine secretions (i.e. histotroph). Given the intimacy of maternal-embryonic nutritional strategies during elasmobranch gestation (Murphy et al., 2006), local trophonematic steroidogenesis and bidirectional exchange of steroid hormones between maternal and uterine compartments are likely.

Little is known regarding histotroph steroid constituents and concentrations in elasmobranchs. Progesterone has been detected in uterine fluid from the Marbled Electric Ray *Torpedo marmorata* (Fasano et al., 1992), a species also utilizing matrotrophic histotrophy;

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however, the stage of pregnancy was not reported. Thus, the objective of this study was to investigate how steroid hormones change over the course of pregnancy within maternal (plasma) and histotroph (uterine fluid) compartments. Specifically, we were interested in the alignment and timing of histotroph concentrations with developmental events (i.e. sexual differentiation, absorption of yolk sacs), and the association, if any, between maternal plasma and histotroph steroid hormone concentrations. Based on established patterns in mammalian pregnancy, we hypothesized that early in development, histotroph hormones would reflect maternal plasma, but that sexual differentiation would be associated with embryonic steroidogenesis.

2. Methods

2.1. Study area

As part of a larger study of the impact of legacy PCB contamination on Round Stingray reproductive success (Lyons and Wynne-Edwards, 2018a), under University of Calgary Animal Care Protocol #14-0016, female stingrays were sampled from two locations in southern California, USA: anthropogenically impacted mainland California at the Seal Beach National Wildlife Refuge (SBNWR, 33.731 N, 118.064 W; hereafter "contaminated" site) and 35 km offshore in Catalina Harbor, Catalina Island (33.434 N, 118.503 W) with much lower anthropogenic impact (hereafter "reference" site). Females were sampled approximately monthly between June and September of 2014 to encompass female pregnancy from post-ovulation to late pregnancy (Jirik and Lowe, 2012) at both sites. There was no dilution of histotroph hormones as uterine flushing did not occur during any captures.

2.2. Legacy PCB contamination

The sites differ in legacy polychlorinated biphenyl contamination (Lyons et al., 2014; Sawyna et al., 2017; Lyons and Wynne-Edwards, 2018a). Other potent endocrine disrupting chemicals found in southern California such as dichlorodiphenyltrichloroethane and pharmaceuticals, are low or non-detectable based on previous work (Lyons et al., 2014, 2018), presumably because the stingrays live near-shore, whereas urban sewage outfall is directed about 8 km offshore at a depth greater than 60 m[1].

2.3. Site effects on embryonic growth

Relative to the reference population offshore, the mainland site impacted by legacy PCB contamination had impaired embryo growth (Lyons and Wynne-Edwards, 2018a). Specifically, in spite of earlier ovulation at the mainland site, embryos from that impacted site grew more slowly, and were smaller at every developmental stage, than embryos at the island site. Developmental alignment of the two sites using "clasper days" (i.e. days relative to male clasper development), showed that differences in embryo mass between sites were also sex-specific, with PCB-exposed embryonic males significantly lighter than their reference (island) counterparts whereas embryonic females showed no differences (Lyons and Wynne-Edwards, 2018a). For these reasons, site was included as a variable in these analyses.

2.4. Site effects on the acute stress response

All adult stingrays were captured via hook and line and subjected to either rapid capture or prolonged capture stress involving time fighting the line, plus confinement for 15 min after dehooking (Lyons and Wynne-Edwards, 2018b). The short duration of the stressor was not expected to alter blood or histotroph reproductive steroid hormone levels. Initial models confirmed the absence of an effect of capture stress. Therefore, stress was not included as a class variable for these analyses. The dominant elasmobranch glucocorticoid, 1α-OH-corticosterone, did mount a response to the stressor; however, elements of glucose mobilization for a secondary stress response were impaired at the contaminated site, particularly for adult males (Lyons and Wynne-Edwards, 2018b). Thus, the effect of site on stress reactivity was another rationale for retaining site as a variable in these analyses.

2.5. Sampling procedure

After capture, stingrays were euthanized with an overdose of tricaine methanesulfonate (MS-222). Prior to tissue sampling, disk width (size measured as the wing tip to wing tip width across the body of the animal) of all stingrays was taken. Adult stingray blood samples were obtained via cardiac puncture, transferred to a heparinized tube, and stored on ice until components could be separated via centrifugation at the lab. Plasma was then stored at −80 °C until analysis. After blood sampling in females, the skin over the ventral side of the body was carefully opened and internal organs (i.e. liver and digestive system) were excised to expose both uteri. A small slit was made in the left uterine wall and a 10 mL syringe with an 18 gauge needle was used to aspirate as much histotroph as possible. After transfer to a 15 mL tube, histotroph was immediately frozen on dry ice and subsequently stored at −80 °C until analysis. Embryos were then removed from both left and right uteri, individually wrapped in aluminum foil, and frozen for later dissection, where the sex (determined by the presence or absence of visible claspers, the male copulatory organ), disk width, total body mass and weight of yolk sac (if present) were recorded. The uterus of origin, unfortunately, was not recorded for individual embryos. No attempt was made to sample embryonic blood or yolk.

2.6. Hormone quantitation

1α-OH-corticosterone is the predominant stress response glucocorticoid used by elasmobranchs (Anderson, 2012). A validated corticosterone ELISA (Evans et al., 2010) found the expected increase in response to the capture stressor, but no effect of stage of pregnancy (Lyons and Wynne-Edwards, 2018b). In the absence of a validated LC-MS/MS method for quantitation of 1α-OH-corticosterone (Wheaton et al., 2018), it was not quantified in histotroph and therefore is not included in these analyses.

2.6.1. Chemicals and reagents

Cortisol, cortisone, corticosterone, 11-deoxycortisol, 11-dehydrocorticosterone, testosterone, 17-hydroxyprogesterone, androstenedione, progesterone, 11-ketotestosterone, estrone, estradiol and estriol were purchased from Steraloids Inc (Newport, RI). Deuterium-labeled internal standards: cortisol-d4, corticosterone-d8, testosterone-d2, 17-hydroxyprogesterone-d8 and progesterone-d9, estrone-2,4,16,16-d4, 17β-estradiol-2,4,16,16-d4 (estradiol-d4), 16α-hydroxy-17β-estradiol-2,4-d2 (estriol-d2) were obtained from CDN Isotopes Inc (Pointe-Claire, Quebec, Canada). HPLC grade methanol, Optima grade acetonitrile and Optima grade water were purchased from Fisher Scientific (Edmonton, AB, Canada).

2.6.2. Sample preparation procedure

A 50 μL sample of maternal plasma or histotroph was transferred into a 0.5 mL micro-centrifuge tube, followed by 50 μL of protein precipitation solution ($ZnSO_4 \cdot 7H_2O$ at 9 mg/mL, in methanol spiked with deuterated internal standards [IS]). After 20 min of cold incubation (4 °C), the mixture was vortexed for 15 s, centrifuged at 14,000 rpm for 15 mins, and the 50% water/50% methanol supernatant (75 μL) was submitted for LC-MS analysis.

[1] https://www.ocsd.com/services/regional-sewer-service.

2.6.3. LC-ESI/MRM analysis

Pooled samples of adult male and female plasma and maternal histotroph were used to establish calibration ranges and as quality control (QC) in each sample matrix, respectively. All samples were analyzed using an Agilent 1200 binary liquid chromatography (LC) system coupled to an AB SCIEX QTRAP® 5500 tandem mass spectrometer equipped with an electrospray ionization (ESI) source. LC separation was performed on an Agilent ZORBAX Eclipse plus C18 column (100 × 2.1 mm, 1.8 µm particle size) at 40 °C for Positive ESI or Agilent Poroshell 120 C18 column (50 × 2.1 mm, 2.7 µm particle size) at 35 °C for Negative ESI. The mobile phase A was acetonitrile (ACN)/H_2O (5/95, v/v, 2 mM NaF) and the mobile phase B was 100% ACN (2 mM NaF). The 12 min gradient for Positive ESI mode was 15–70% B (0–6 min), 70–100% B (6–7 min), 100% B (7–8.5 min), 100–15% B (8.5–9 min), held at 15% B for 3 min. The 9 min gradient for Negative ESI mode (estrogens) was 40–80% B (0–4 min), 80–100% B (4–4.5 min), 100% B (4.5–5.5 min), 100–40% B (5.5–6 min), held at 15% B for 3 min. The flow rate was 0.4 mL/min and the injection volume was 12 µL. Mass resolutions in Q1 and Q3 were set to unit resolution. Each analyte was monitored by two transitions (a quantifier and a qualifier; Table 1).

2.6.4. Quantitation method

Steroids were quantified as area ratio relative to the bio-identical deuterated internal standard. This approach to quantification corrects for matrix influences on extraction efficiency within samples. Calibration curves used analyte/IS peak area ratios (y-axis) vs. analyte concentration (x-axis) and a linear fit with 1/x weighting. Fit (i.e. r^2) for all calibration curves were always higher than 0.995, and typically higher than 0.998. The lowest concentration of a steroid that gives < 20% coefficient of variation (CV) and < ± 30% error was determined as its lower limit of quantification (Table 1). Three quality control solutions were run in duplicate. At the lowest QC tested (0.25 or 0.5 ng/mL), duplicate CV for individual steroids (mean CV across steroids = 5.72%) ranged from 0.1% (deoxycortisol) through 15.1% (estradiol). Quantified hormone concentrations (ng/mL) were converted to nmol/L prior to analyses.

2.7. Data analysis

R statistical package (V 3.4.1) (Team RDC, 2011) was used for all analyses and α was set to 0.05. Mean ± the variance is represented as standard deviation (SD), and % CV (SD/mean * 100) is reported for quantitation duplicates. Given the previously established reproductive asynchrony between populations (Lyons and Wynne-Edwards, 2018a), litters were grouped based on developmental stage (a discrete variable) and age relative to sexual differentiation (clasper days, a continuous variable) to compare hormone concentrations. For developmental stage, litters were assigned to one of the following groups based on the origin of supplemental nutrition: post-ovulation (no visible embryos), early-term (yolk-dependent embryos), mid-term (partially yolk-dependent, partially histotroph-dependent embryos), and late-term (histotroph-dependent embryos). For sexual differentiation, embryos were aligned against clasper days (Lyons and Wynne-Edwards, 2018a). With these two approaches, we investigated hormone changes with respect to yolk utilization as well as sexual differentiation. Hormonal differences were explored using multiple comparisons tests and linear regressions, respectively. Maternal and embryonic compartments were examined separately before pairwise comparisons of plasma and histotroph were conducted using Pearson's correlations and paired t-tests.

No attempt was made to quantify yolk steroids. However, hormone concentrations were compared relative to yolk sac mass and embryo mass. Yolk sac size was expressed as a percentage of total embryo mass (i.e. [yolk sac weight]/[yolk sac weight + embryo weight] * 100).

Besides changes with development, other factors such as litter sex composition or embryo number could influence histotroph concentrations. Unfortunately, embryo position (i.e. right or left uterus) was not recorded and the anatomic expectation (Spieler et al., 2013) is that uterine fluid cannot be exchanged between uteri. Therefore, tests of effect of sex on histotroph steroid concentration used only the subset of litters that were single sex. We hypothesized that male-only litters would have greater testosterone and lower estradiol concentrations than female-only litters based on mammalian studies (vom Saal, 1981).

3. Results

Maternal plasma concentrations of reproductive hormones did not differ between sites when compared either by embryonic stage (all $p \geq 0.10$) or date relative to male embryonic clasper formation ("clasper days"; $p = 0.47$). Therefore, sites were combined for subsequent analyses. Our resulting pregnant female sample set (n = 55, sites combined) was comprised of post-ovulatory (n = 6), early- (n = 11), mid- (n = 14) and late-term (n = 24) females. Females were not sampled before ovulation. Six females aborted their litters during euthanasia and before histotroph was sampled and thereby reduced the sample size to 49 for histotroph and maternal-histotroph paired analyses.

Pooled samples of plasma (containing both adult males and adult females) and histotroph (adult females only) were run in triplicate for quality control assessment. Only cortisol and testosterone were quantifiable in pooled adult plasma (cortisol CV = 7.0%; testosterone CV = 6.1%). In pooled histotroph, cortisol was below the lower limit of quantitation (LLOQ), but testosterone (CV = 4.0%), 17-hydroxyprogesterone (CV = 9.7), progesterone (CV = 4.6%), estriol

Table 1
Quantifier and qualifier mass transitions (*m/z*) from the parent (left of /) to daughter (right of /) h for each steroid and deuterated internal standard monitored. The LLOQ with this multiple steroid MRM method is also shown. The total number of deuterium substitutions in the internal standard is shown in parentheses for each analyte.

Analyte	Transitions (non-deuterated)	Transitions (deuterated)	LLOQ (ng/mL)
11-deoxycortisol	347/97, 347/109	–	0.05
Cortisol (d4)	363/121, 363/327	367/121, 367/331	0.1
11-dehydrocorticosterone	345/121,354/301	–	0.05
Corticosterone (d8)	347/329, 347/121	355/337, 355/125	0.05
Cortisone	361/163, 361/121	–	0.05
Testosterone (d2)	289/91, 389/109	291/99, 291/111	0.05
11-ketotestosterone	303/121, 303/259	–	0.1
17-hydroxyprogesterone (d8)	331/97, 331/109	339/100, 339/109	0.05
Progesterone (d9)	315/97, 315/109	324/100, 324/113	0.05
Androstenedione	287/97, 287/109	–	0.05
Estriol (d2)	287/171, 287/145	289/173, 289/147	0.1
Estradiol (d4)	271/145, 271/183	275/147, 275/187	0.05
Estrone (d2)	269/145, 269/143	273/147, 273/145	0.05

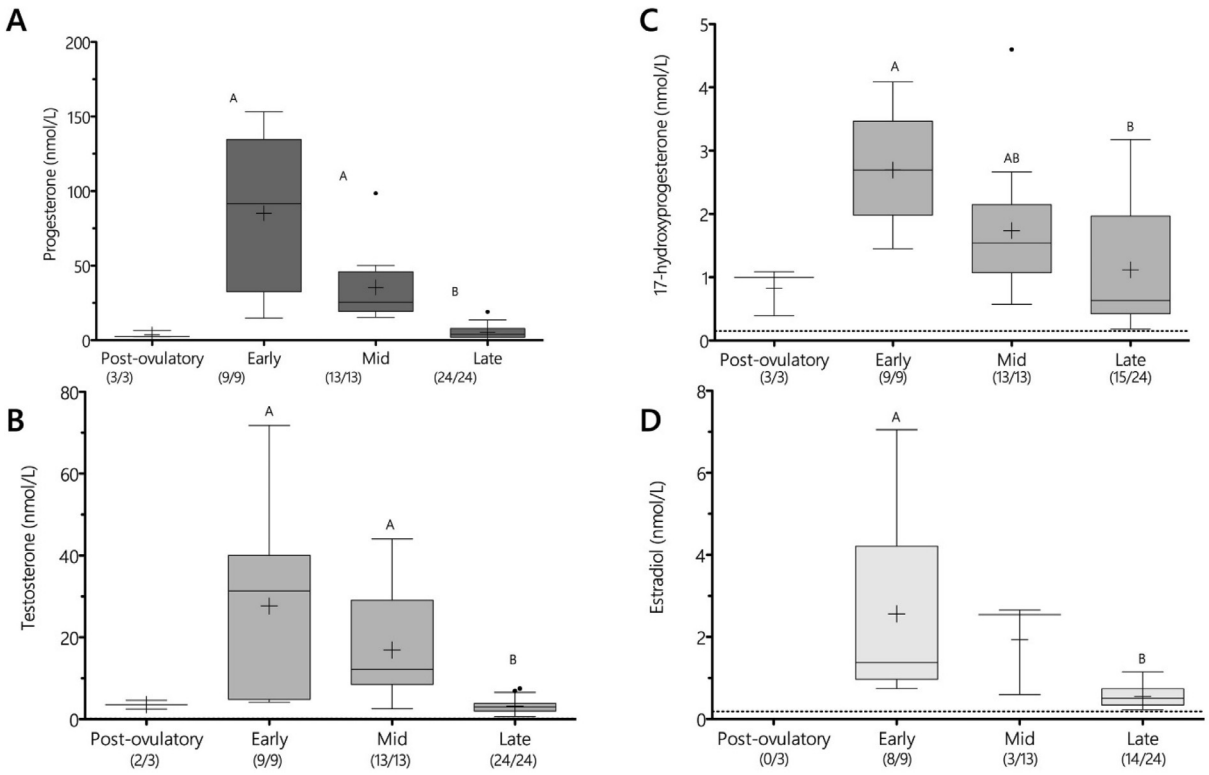

Fig. 1. Histotroph concentrations of progesterone (A), testosterone (B), 17-hydroxyprogesterone (C) and estradiol (D) changed with embryonic developmental stage. Different letters denote significant differences among groups ($\alpha < 0.05$). The absence of a letter indicates groups with too few samples for statistical comparison. Dashed horizontal line indicates lower limit of quantitation for each hormone.

(CV = 3.7%) and estradiol (CV = 2.5%) were reliably quantifiable. Cortisone, 11-deoxycortisol, corticosterone, 11-dehydrocorticosterone, and 11-ketotestosterone were below LLOQ in pooled samples of plasma and of histotroph.

3.1. Maternal plasma

As predicted by the pooled plasma steroid quantitation, testosterone was consistently detected in maternal plasma (54/55, 98%; for adult male plasma testosterone, see Supplemental Fig. 1). Estradiol (17/55, 31%), progesterone (7/55, 13%), and 11-dehydrocorticosterone (4/55, 7%) were detected less frequently. Unlike the pooled plasma, cortisol was rarely quantifiable in maternal plasma (5/55, 9%), which is likely due to the inclusion of one male sample with high cortisol (see supplemental materials in Lyons and Wynne-Edwards, 2018b). Additional hormones quantified in this study were not above LOQ in any maternal plasma samples.

Maternal plasma testosterone was the only hormone to show development-related changes in concentration (KW, W = 23.6, df = 3, $p < 0.0001$). Testosterone concentrations increased from post-ovulation to peak mid-term before subsequently decreasing in late-term females (Table 2).

Estradiol was most frequently detected in post-ovulatory females (3/6, 50%) followed by late (9/24, 37.5%) and early (4/11, 36%) term females, with only one mid-term detection (n = 14, 7%). When quantifiable, estradiol increased from early (0.24 ± 0.049 nmol/L) to late-term pregnancy (0.44 ± 0.32 nmol/L; p = 0.05). Progesterone had single detections in both the post-ovulatory and early-term time periods, with the majority of detections occurring during the late term (5/24, 21%). Progesterone was highest in a post-ovulatory female (1.14 nmol/L), but similar between an early-term (0.19 nmol/L) and late-term females (0.18 ± 0.028 nmol/L).

Cortisol was only detected in post-ovulatory females (4/6, 66%) and one early-term female, whereas 11-dehydrocorticosterone was only detected in late term females (4/24, 17%).

3.2. Histotroph

Confirming the histotroph findings in the Marbled Electric Ray (Fasano et al., 1992), progesterone was quantifiable in every sample (49/49, 100%). Save for testosterone (48/49, 98%), which was comparable in terms of detection frequency to maternal plasma, hormones generally had greater detection rates in histotroph than maternal plasma. 17-hydroxyprogesterone (41/49, 84%), estriol (28/49, 57%), and estradiol (25/49, 51%) were quantifiable throughout pregnancy. On the other hand, androstenedione (13/49, 27%) and estrone (6/49, 12%) were detected in only early and mid-term samples, with the former measured in all nine early-term litters. With respect to corticosteroids, 11-deoxycortisol, the immediate precursor of cortisol in the synthesis pathway, was detected throughout pregnancy (15/49, 31%), while “endpoint” corticosteroids (i.e. corticosterone and cortisol) were detected in mid and late term litters (7/49, 14% and 2/49, 4%, respectively; Table 2).

Only four hormones showed concentration changes with development (progesterone, testosterone, 17-hydroxyprogesterone, and estradiol) and there were few effects of site. With respect to developmental stage, patterns of increase and decrease were most similar between progesterone and testosterone (Fig. 1A, B). In reference litters, progesterone and testosterone concentrations increased from post-ovulation to peak in early-term litters, followed by a sharp concentration decrease in mid-term litters that persisted into the late term (P: W = 12.9, df = 2, p = 0.0015; T: W = 7.48, df = 2, p = 0.024). Progesterone and testosterone in PCB-exposed litters followed a similar pattern, with concentrations peaking in the early term followed by a significant decrease in mid- and late-term litters (P: W = 19.9, df = 2, $p < 0.0001$; T: W = 15.6, df = 2, $p < 0.0005$). 17-

Table 2
Mean ± SD concentrations (nmol/L) histotroph steroids by stage of pregnancy are presented for litters where concentrations were above detection limits (number of quantifiable detections for each group shown in parentheses). As the only consistently detected maternal plasma steroid, maternal plasma testosterone is also presented for comparison in [square] brackets. Litters were divided into four developmental stages (sites combined): post-ovulatory (yolk, no visible embryos), early-term (embryos fully dependent on yolks), mid-term (embryos exhausting yolks), and late-term (embryos fully histotroph dependent). Number of pregnant females and associated histotroph collected is given for each group. Cortisone and 11-ketotestosterone were not detected in any samples.

	Post-ovulatory	Early-term	Mid-term	Late-term
Cortisol	–	–	–	0.50–0.69 (2)
11-deoxycortisol	–	0.20 ± 0.047 (7)	0.27 ± 0.16 (4)	0.22 ± 0.06 (4)
11-dehydrocorticosterone	–	–	–	0.36 (1)
Corticosterone	–	–	0.57 ± 0.13 (3)	2.75 ± 2.22 (4)
Testosterone	3.54 ± 1.52 (2)	27.7 ± 22.5 (9)	16.9 ± 12.5 (13)	3.30 ± 1.87 (24)
[maternal plasma T]	[0.924 ± 0.698 (6)]	[2.14 ± 1.11 (10)]	[4.35 ± 1.51 (14)]	[2.42 ± 1.31 (24)]
17-hydroxyprogesterone	0.83 ± 0.38 (3)	2.70 ± 0.86 (9)	1.74 ± 1.06 (13)	1.09 ± 0.90 (16)
Androstenedione	–	0.68 ± 0.43 (9)	0.42 ± 0.36 (3)	0.17 (1)
Progesterone	3.69 ± 2.40 (3)	85.2 ± 50.9 (9)	35.4 ± 22.4 (13)	4.82 ± 3.81 (24)
Estriol	–	1.023 ± 0.71 (5)	0.42 ± 0.24 (2)	0.65 ± 0.38 (21)
Estradiol	–	2.56 ± 2.26 (8)	1.93 ± 1.16 (3)	0.55 ± 0.26 (14)
Estrone	–	0.46 ± 0.21 (5)	0.19 (1)	–
#Pregnant females	6	11	14	24
#Histotroph sampled	3	9	13	24

hydroxprogesterone also peaked in early-term litters but concentrations decreased more gradually as mid-term concentrations were not significantly different than late-term at either site (Reference: $W = 5.78$, $df = 2$, $p = 0.055$; PCB-exposed: $W = 11.6$, $df = 2$, $p = 0.003$; Fig. 1C). Despite estradiol being detected less consistently, concentrations showed similar patterns as the other three hormones with highest concentration in the early-term and significantly lower concentrations in the mid- and late-term litters (sites combined due to low detection rates: $W = 13.5$, $df = 2$, $p = 0.0012$; Fig. 1D). Therefore, histotroph steroid composition may reflect embryonic sexual differentiation.

These four hormones also showed developmental changes respect to sexual differentiation (clasper days), a continuous variable (Fig. 2A–D). Male and female embryos were externally distinguishable at a small size (approximately 3.2% and 3.5% of final mass), indicating that sexual differentiation occurs during the early-term stage. Hormone concentrations were highest early in development just before or at the first appearance of claspers (i.e. clasper day 0), and significantly declined as development progressed for both sites (all $p \leq 0.018$). While the pattern was the similar between sites, there was a significant effect of site for all four hormones (all $p \leq 0.04$) and a significant interaction for progesterone, testosterone and estradiol (all $p \leq 0.039$). This was due to the higher concentrations in contaminated embryos just prior to sexual differentiation compared to the reference site where the smallest litters were already sexually differentiated (i.e. clasper day 0). The peak in hormone concentrations around the time of clasper appearance (or lack thereof in females), implicates embryo sexual differentiation as a developmental landmark for these changes in histotroph steroid hormone environment during early development.

During early development, embryo yolk sacs were rapidly exhausted and were completely absent after embryos had reached ~20% of their final mass. As yolk sacs were depleted, concentrations of progesterone and testosterone increased in histotroph during early development (Fig. 3).

3.3. Maternal vs. embryo

As testosterone was consistently detected in plasma and histotroph, we used it to identify hormone sources and sinks over development. Matched pairs of maternal plasma and histotroph showed stage-specific linear associations (Fig. 4). No correlations existed for early-term litters ($p = 0.1$), but samples were correlated in mid-term litters ($p = 0.019$, $r = 0.58$). During late-development, plasma-histotroph pairs remained correlated ($p = 0.043$, $r = 0.36$). No correlations existed between plasma and histotroph progesterone and estradiol, regardless of stage. Contrary to predictions, histotroph testosterone was significantly higher than its corresponding plasma pair for early ($t_7 = 3.0$, $p = 0.019$), mid ($t_{12} = 3.9$, $p = 0.002$), and late-term ($t_{22} = 2.4$, p 0.035) litters (Fig. 5). Dividing litters by sexual differentiation led to similar findings of histotroph having significantly higher concentrations than paired plasma (pre: $t_7 = 2.6$, $p = 0.034$; post: $t_{37} = 3.3$, $p = 0.002$). Testosterone peaks in each respective tissue also differed (Fig. 5). Histotroph preceded plasma and peaked during early-term development, whereas maternal plasma testosterone did not peak until the mid-term.

To examine the influence of sex, litters that had only males or females ($n = 5$ and 6, respectively) were compared. Previous work noted site-related differences between male stingray embryos (Lyons and Wynne-Edwards, 2018a). Unfortunately, low sample size prevented us from detecting any effect of site. Testosterone was comparable in early term litters regardless of sex (Fig. 6A) as was progesterone. Testosterone decreased more steeply in female-only litters after the normal point of male sexual differentiation. Conversely, estradiol and estriol were detected in female-only litters much earlier than in male-only litters (Fig. 6B). When estrogens were detected in male-only litters, concentrations were generally comparable to that of females. The earlier presence of estradiol in female-only litters suggests that both male and female embryos influence histotroph steroid composition.

4. Discussion

4.1. Maternal plasma steroids

Testosterone was consistently detected in maternal plasma throughout gestation. The presence of testosterone in other elasmobranch females, utilizing placentrotrophy, is noted to peak around mating and ovulation (Manire et al., 1995). These latter reproductive milestones were not sampled in the present study; however, we found female stingray testosterone peaked later than previous elasmobranch studies. The increase in testosterone later in pregnancy might be due to uterine influences on plasma (see Section 4.3), and differences in matrotrophic strategy between Round Stingrays and placental sharks, which could influence when testosterone peaks appear.

In both populations, a variety of corticosteroids were quantified, including one that has not been reported in elasmobranchs (11-deoxycortisol), despite it being the main corticosteroid of the sea lamprey *Petromyzon marinus* (Close et al., 2010), a jawless fish. For females, studies have noted increases in corticosteroids during ovulation in other elasmobranchs (Manire et al., 2007), and likely account for the presence of cortisol in reference female stingrays post-ovulation. Our inability to sample ovulating females at the PCB-contaminated site

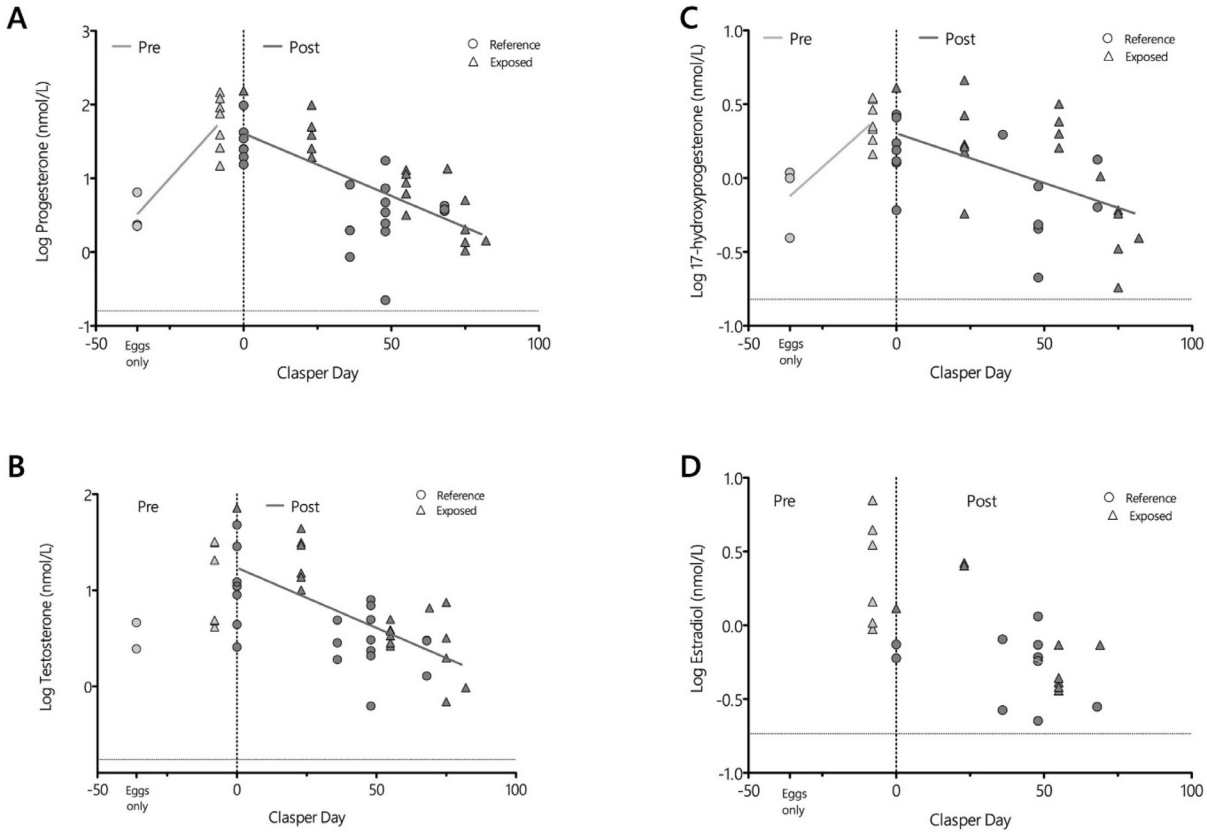

Fig. 2. Histotroph steroid changes at the time of external sexual differentiation (male clasper formation). To visualize how histotroph hormone concentrations changed with respect to sexual differentiation, litters were divided into pre- (light grey) and post- (dark grey) groups and aligned between sites using clasper days as a developmental marker. Linear regressions were performed with reference (circles) and PCB-exposed ("exposed", triangles) pooled for simplicity, with significant relationships depicted (solid regression lines). The first appearance of claspers on male embryos (0 clasper day) is denoted by a vertical dashed line, while a dashed horizontal line indicates lower limit of quantitation (LLOQ) for each hormone. Note that all values are on logarithmic scales.

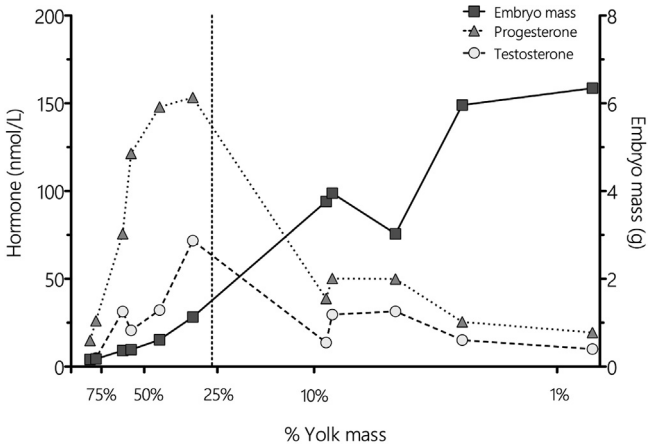

Fig. 3. Relationship of relative yolk mass size to histotroph progesterone and testosterone concentrations (left y-axis) and embryo mass (right y-axis)The absorbance of yolk sacs over early pregnancy, depicted as the contribution of yolk mass to total embryo plus yolk mass, is compared to histotroph concentration changes of progesterone (triangle) and testosterone (circle) and embryo mass without yolk mass (squares). Dashed vertical line denotes male embryo external sexual differentiation (male clasper appearance).

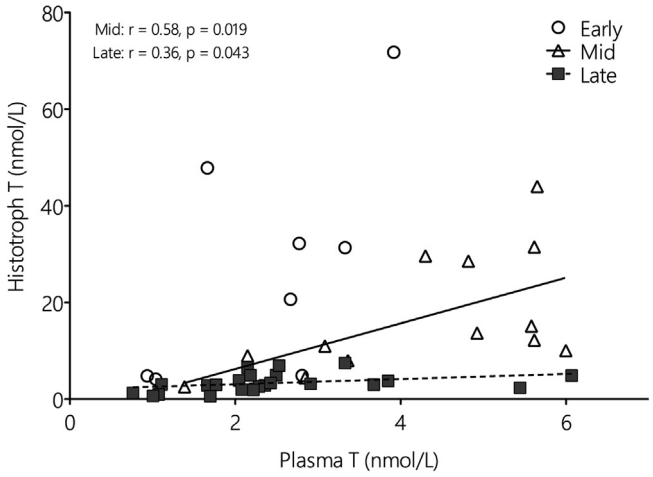

Fig. 4. Associations between maternal plasma and histotroph testosterone (sites combined). Testosterone in plasma and histotroph pairs were not correlated in early-term litters ($p = 0.1$, open circles), but were correlated ($p < 0.02$) during the mid-term (grey triangles, solid line) when plasma testosterone peaked. In late-term development (black squares, dashed line), a correlation persisted between plasma and histotroph pairs ($p = 0.04$).

prevented us from making site comparisons.

4.2. Mothers: hormone sink or source?

The low concentrations and detection rate of hormones in plasma was unexpected, considering that, in other elasmobranchs, concentrations of sex steroids tend to peak around the time of ovulation and are detectable throughout pregnancy (Manire et al., 1995; Tricas et al., 2000; Mull et al., 2010). The reduced number of detections could be related to the high specificity of LC-MS/MS or the sensitivity of our

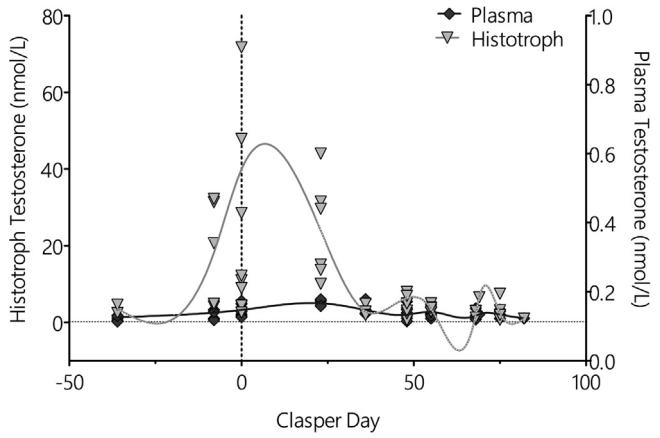

Fig. 5. In all developmental stages (sites combined), histotroph (grey triangles/line) had significantly higher concentrations than plasma (black diamonds/line) pairs. Histotroph concentrations peaked prior to plasma. Cubic spline curves were fitted to the data to depict concentration changes with development. Among possible sources of error is that testosterone concentrations could not be corrected for the number of male embryos in the left uterus, where the histotroph was collected.

method (0.05–0.1 ng/mL) compared to other studies (3 pg/mL; Manire et al., 2007). Nevertheless, histotroph concentrations were higher, and more frequently detected than plasma.

Maternal (plasma) and embryonic (histotroph) compartments have a large surface area of juxtaposition through the villi of the trophonemata that should facilitate diffusion of unbound steroids. Histotroph steroids might cross back into maternal plasma where they could be subject to maternal clearance and excretion. Likewise, maternal steroids should reach the histotroph. In addition, the trophonemata itself might be steroidogenic, as is the case of uterine tissue in mice during early pregnancy (Arensburg et al., 1999), and mammalian placentae (Strauss et al., 1996), and is likely to play a role in modulating steroid transfer between maternal and fetal compartments (Painter et al., 2002). Thus, histotroph steroid composition was not simply a reflection of maternal plasma.

4.3. Yolk precursors and embryonic steroidogenesis

Hormones present in histotroph could result from maternal deposition in yolk and/or from embryonic steroidogenesis from maternal yolk precursors, such as cholesterol (Thompson and Speake, 2002), deposited by mothers during vitellogenesis. Particularly in egg-laying animals, females maternally influence embryonic development through yolk steroids in the absence of a physical maternal-embryo connection (Adkins-Regan et al., 1995; Schwabl, 1996; Conley et al., 1997). In elasmobranchs, some steroid (Manire et al., 2004) and thyroid hormones (triiodothyronine and thyroxine) (McComb et al., 2005) have been reported in the yolks of Bonnethead Sharks (*Sphyrna tiburo*), a placental elasmobranch, from pre-ovulation through early embryonic development. McComb et al. (2005) suggested that increases in yolk thyroid hormones could be due to conversion activities in the yolk as well as possible embryonic production. In birds and reptiles, maternally deposited hormone concentrations even vary among different layers of yolk (Bowden et al., 2001; Moore and Johnston, 2008), which might alter their temporal availability.

Histotroph from post-ovulatory females where only yolk sacs were present (i.e. no visible embryos) had lower concentrations of progesterone, testosterone, 17-hydroxyprogesterone and estradiol than histotroph containing visible embryos. In addition, yolk is membrane bound so that yolk is taken up by embryos before being released into the uterine environment (Conley et al., 1997). As lipid content of Round Stingray yolk is substantially greater than aqueous histotroph (Lyons, unpublished data), steroid solubility would be highest in yolk. Therefore, yolk precursor steroids are likely to provide substrates for early embryonic steroidogenesis.

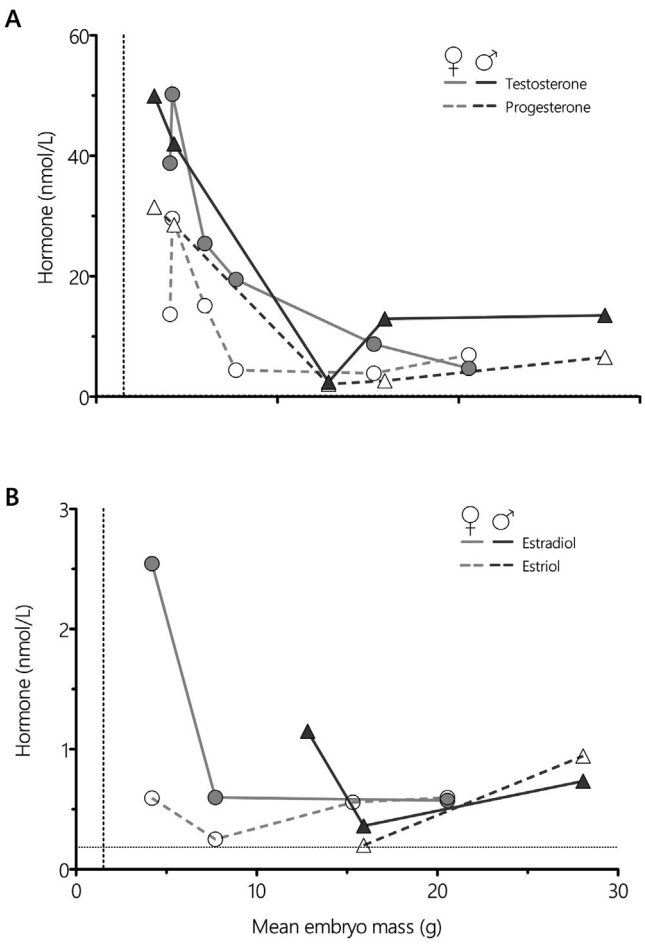

Fig. 6. Sex effect on histotroph steroids using only single-sex litters. To investigate potential sex-related effects on histotroph steroid composition, we compared concentrations of male-only (n = 5, triangles) to female-only (n = 6, circles) mid- to late-term litters. (A) Testosterone (dashed lines) and progesterone (solid line) were similar between the sexes. (B) Estradiol (solid lines) and estriol (dashed lines) were both detected earlier in pregnancy in female-only litters compared to males, suggesting a sex-related effect on histotroph. Vertical dashed line indicates the size at which male embryos could be sexually differentiated, and (B) horizontal lines denote the lower limit of quantitation for estrogens.

Mammalian studies have documented the steroidogenic ability of early stage embryos (Wilson et al., 1980; Stone et al., 1986), which leads to steroids concentrating to greater levels in uterine fluid than in plasma (Rivarola et al., 1968). Swine blastocysts (14–16 days post-fertilization) are steroidogenic and utilize progesterone originating from their mothers to produce androgens and estrogens (Bazer et al., 1979). Many of these observations occurred during a short time frame with high steroidogenic outputs on the part of the embryo, and a similar phenomenon could be occurring in stingray development.

While we did not attempt to quantify steroidogenic capability in embryonic plasma or tissues, the gonad is the likely site of steroid synthesis, based on the high concentrations of sex steroids (e.g. progesterone, testosterone). Steroid hormones originating from other tissues with known steroidogenic activity in adults (i.e. interrenal glands) were detected, particularly 11-deoxycortisol and corticosterone, but their concentrations were much lower and detections less consistent in histotroph. This suggests that either gonadal over interrenal steroidogenesis is more active in stingray embryo development at this time

or that we missed a peak in corticosteroids that might occur early in development as it does in teleost fishes (e.g. de Jesus and Hirano, 1992; Alsop and Vijayan, 2009).

4.4. Role of uterine steroids

In line with other taxa (Janzen et al., 1998) and elasmobranchs (Manire et al., 2004; Hoff, 2009), sexual differentiation occurs very early in Round Stingray development. The peak in estradiol and testosterone just prior to, and with, the appearance of claspers (male copulatory organs), respectively, suggest a role in secondary sexual differentiation. Other intermediary hormones, such as 17-hydroxyprogesterone and androstenedione, may also promote this process (Glickman et al., 1987). While the presence of specific sex chromosomes is ambiguous in elasmobranchs (Maddock and Schwartz, 1996; Souza Valentim et al., 2013), the results of this study suggest that testosterone and estradiol are associated with secondary, and perhaps primary, sexual differentiation.

Besides serving as a substrate for testosterone, estrogen and glucocorticoid steroid synthesis, locally-produced progesterone could have a role facilitating nutrient transfer from mothers to embryos within the uterus. Steroids produced by mammalian embryos can alter uterine secretions to promote nutrient availability (Spencer et al., 2008; Forde et al., 2009), and similar physiological strategies may occur during elasmobranch pregnancies. Low maternal plasma progesterone, at the same time as high histotroph progesterone, supports the possibility of embryonic and trophonemata steroidogenesis. Considering the wide range of matrotrophic strategies among elasmobranchs, future studies should investigate the role of embryonic steroidogenesis in altering their own developmental environment relative to maternal plasma.

4.5. Effects of embryo sex

We did not find any evidence that the sex of the litter influenced testosterone or estradiol concentrations; however, our sample size was small, and not focused around the interval when sex steroid concentrations peaked. The only difference between single-sexed litters was the detection of estradiol and estriol during early development in female-only litters but not in male-only litters. In avian species, with ZW rather than XY genetic sex determination, sexual differentiation is directed by estradiol (reviewed in Smith and Sinclair, 2004), not testosterone as it is in mammalian species (reviewed in Wilhelm et al., 2007). While these results should be interpreted with caution due to the limited number of single-sexed litters available at appropriate times, future research should investigate the role of embryonic steroidogenesis (estrogens or testosterone) in the sexual differentiation of elasmobranchs.

5. Conclusions

Results suggest that Round Stingray embryos alter their uterine steroid environment, with changes closely tied to sexual differentiation early in development. Future studies will need to record embryo sex and number independently for each uterus and collect histotroph from both uteri to match to those embryos. With those data it should be possible to test the hypothesis that male embryos alter the sex steroid environment for females sharing the same uterus (Nagamani et al., 1979), and vice versa. We also recommend projects investigating sex-determining genes to consider screening for those associated with estrogen receptors (O'Shaughnessy et al., 2015), as this may yield promising results. Capitalizing on the diversity of elasmobranch matrotrophic strategies should clarify the role, if any, for maternal-embryonic steroid signals in this ancient taxon.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2018.12.009.

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