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Alterations in eicosanoid composition during embryonic development in the chorioallantoic membrane of the American alligator (*Alligator mississippiensis*) and domestic chicken (*Gallus gallus*)

Theresa M. Cantu[a,b,*], **John A. Bowden**[c], **Jacob Scott**[b], **Jimena B. Pérez-Viscasillas**[d], **Kevin Huncik**[c], **Matthew P. Guillette**[a,b], and **Louis J. Guillette Jr.**[a,b]

[a]Medical University of South Carolina, Department of Obstetrics and Gynecology, 331 Fort Johnson Road, Charleston, SC 29412, United States

[b]Hollings Marine Laboratory, 331 Fort Johnson Road, Charleston, SC 29412, United States

[c]National Institute of Standards and Technology, Chemical Sciences Division, Environmental Chemical Sciences Group, Hollings Marine Laboratory, 331 Fort Johnson Road, Charleston, SC 29412, United States

[d]Grice Marine Laboratory, College of Charleston, 205 Fort Johnson Rd, Charleston, SC 29412, United States

Abstract

Eicosanoids are signaling lipids known to regulate several physiological processes in the mammalian placenta, including the initiation of parturition. Though all amniotes construct similar extraembryonic membranes during development, the composition and function of eicosanoids in extraembryonic membranes of oviparous reptiles is largely unknown. The majority of effort placed in eicosanoid investigations is typically targeted toward defining the role of specific compounds in disease etiology; however, comprehensive characterization of several pathways in eicosanoid synthesis during development is also needed to better understand the complex role of these lipids in comparative species. To this end, we have examined the chorioallantoic membrane (CAM) of the American alligator (*Alligator mississippiensis*) and domestic chicken (*Gallus gallus*) during development. Previously, our lab has demonstrated that the CAM of several oviparous species shared conserved steroidogenic activity, a feature originally attributed to mammalian amniotes. To further explore this, we have developed a liquid chromatography/tandem mass spectrometry method that is used here to quantify multiple eicosanoids in the CAM of two oviparous species at different stages of development. We identified 18 eicosanoids in the alligator CAM; the cyclooxygenase (COX) pathway showed the largest increase from early development to later development in the alligator CAM. Similarly, the chicken CAM had an increase in COX products and COX activity, which supports the LC-MS/MS analyses. Jointly, our findings indicate that the CAM tissue of an oviparous species is capable of eicosanoid synthesis, which expands our

*Corresponding author at: Medical University of South Carolina, Department of Obstetrics and Gynecology, 331 Fort Johnson Rd. Charleston, SC 29412, United States. theresacantu23@gmail.com (T.M. Cantu).

knowledge of placental evolution and introduces the possibility of future comparative models of placental function.

Keywords

American alligator; Eicosanoid; Prostaglandin; Chorioallantoic membrane; Oviparous; Amniotes; Cyclooxygenase

1. Introduction

Eicosanoids, oxidized lipids derived from the metabolism of polyunsaturated fatty acids (PUFAs), are largely known for their role in inflammation (e.g., arthritis, asthma) and immune system response. Within the endocrine system, eicosanoids function as “local” hormones and mediate several biological processes through both autocrine and paracrine signaling. These signaling lipids are diverse in structure and function, illustrated by the fact that certain members of this family both initiate as well as resolve inflammation (prostaglandin E_2 and resolvin D_1, respectively). Due to this diversity, methods aimed at measuring eicosanoids on a comprehensive level are now utilized as a systems biology approach to investigate how these bioactive lipids function at the cellular and organismal level. Of particular interest to our laboratory are the roles of these lipids in various reproductive and developmental processes (Bergström et al., 1968; Hertelendy and Zakár, 2004; Horton, 1969). In mammalian species, certain eicosanoids, such as prostaglandin E_2 and $F_{2\alpha}$, are known to function as local mediators that work in several signaling processes, including the initiation of parturition (Cook et al., 2014; Olson, 2003) and induction and resolution of inflammation (Serhan, 2014; Serhan et al., 2015a,b). Specifically, within the mammalian placenta, these lipid mediators are essential for the maintenance of pregnancy, initiation of uterine contractions and lung maturation in the fetus (Alvaro et al., 2004; Keelan et al., 2003; Sales and Jabbour, 2003; Thorburn, 1991; Weems et al., 2006). Though these lipid mediators are extensively studied in both basic immunology and within certain placental pathologies in mammals (e.g., preeclampsia, preterm birth), few studies have focused on examining the presence and the role of lipid mediators in non-mammalian extraembryonic membranes during embryonic development (Challis et al., 2002; Friedman, 1988; Maynard and Karumanchi, 2011; Walsh, 2004). In this study, we examined the eicosanoid milieu of the chorioallantoic membrane (CAM) in two non-mammalian amniotes, the American alligator (*Alligator mississippiensis*) and domestic chicken (*Gallus gallus*), to investigate the potential conserved eicosanoid activity of the CAM in the progenitor to the mammalian placenta.

All amniotes share the same extraembryonic membranes, which include the amnion, yolk sac, and chorioallantois (Kluge, 1977). In oviparous species (egg-laying), the latter is known as the chorioallantoic membrane (CAM) (Thompson and Speake, 2006). In viviparous species (live-bearing) this membrane, in concert with the mother’s uterine tissue, is known as the chorioallantoic placenta (Carter and Mess, 2007; Cross et al., 2003; Wildman et al., 2006). Both membranes of oviparous and viviparous species share similarities in origin, structure, and function, which include water and gas exchange, nutrition, and waste removal

(Kluge, 1977). Additionally, the chorioallantoic placenta exhibits endocrine capabilities in viviparous species, a characteristic originally hypothesized as a novel adaptation of the eutherian organ (Cross et al., 2003). Recently, it was discovered that the CAM of the domestic chicken (*Gallus gallus*), an oviparous species, displayed endocrine activity, with noted roles in steroid synthesis and signaling (Albergotti et al., 2009). The latter study was further supported by an examination of another oviparous species, which noted that the CAM of the American alligator also had steroidal activity (Cruze et al., 2012). Collectively, these studies support the hypothesis that endocrine function in extraembryonic membranes is a conserved characteristic among all amniotes, not exclusive to eutherians (Cruze et al., 2012).

Following the hypothesis that certain aspects of endocrine activity in extraembryonic membranes are a shared characteristic among all amniotes (oviparous and viviparous), we hypothesize that eicosanoid activity is conserved in the chorioallantoic membrane of oviparous amniotes. If eicosanoids were found in the oviparous CAM, it would provide further evidence that immune function is also potentially conserved in this tissue and thus would offer another comparative model for studying placental function. However, to expand on the analyses of steroid hormone activity in the CAM and further investigate the possible immune function of this tissue, we must first characterize the eicosanoid composition during embryonic development among different oviparous species. To do this, we have created a comprehensive, direct method for the measurement of the eicosanoid milieu in both the alligator and chicken CAM tissue and investigated cyclooxygenase (COX) activity in the avian CAM. The current method of choice for quantitative eicosanoid profiling is achieved through the use of liquid chromatography/tandem mass spectrometry (LC-MS/MS) (Astarita et al., 2015; Capra et al., 2014; Masoodi et al., 2010). Though this technology has been used frequently in clinical studies and cell work, it is particularly useful for non-traditional species, as it does not require antibodies for measurement of eicosanoids. The main objectives of this investigation were to (1) create a new method for the quantification of several different classes of eicosanoids in the CAM tissue of two oviparous species, (2) examine the composition of eicosanoids during embryonic development in this tissue, and (3) analyze the cyclooxygenase activity in the avian CAM to compliment results from the developed LC-MS/MS method. Combined, these analyses provide evidence of eicosanoid activity in the reptilian and avian CAM, and propose that immune function in this tissue is potentially conserved across all amniotes.

2. Methods

2.1. Chemicals

For the purpose of method development, we chose to focus on six different classes of eicosanoids – as shown in Fig. 1. The eicosanoid standards used were prostaglandin $F_{2\alpha}$, prostaglandin E_2, prostaglandin D_2, 6-keto prostaglandin $F_{1\alpha}$, thromboxane B_2, (±)1 1,12-dihydroxy-5Z,8Z,14Z-eicosatrienoic acid (±11,12-DHET), (±)1 4,15-dihydroxy-5Z,8Z,11Z-eicosatrienoic acid (±14,15-DHET), (±)1 1(12)-epoxy-5Z,8Z,14Z-eicosatrienoic acid (11(12)-EET), (±)14(15) -epoxy-5Z,8Z,11Z-eicosatrienoic acid (14(15)-EET), (±)13S-hydrox y-9Z,11E-octadecadienoic acid (±13(S)-HODE), (±)-9-hydroxy-10E, 12Z-

octadecadienoic acid (±9(S)-HODE), 5S-hydroxy-6E,8Z,11Z,14 Z-eicosatetraenoic acid (5(S)-HETE), 15S-hydroxy-5Z,8Z,11Z,13E-e icosatetraenoic acid (15(S)-HETE), 12S-hydroxy-5Z,8Z,10E,14Z-eic osatetraenoic acid (12(S)-HETE), (±)11-hydroxy-5Z,8Z,12E, 14Z-eic osatetraenoic acid (±11-HETE), and were purchased from Cayman Chemical (Ann Arbor, MI) with a purity above 97%. Arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), docosapentaenoic acid n-3, docosapentaenoic acid n-6, and adrenic acid were purchased from Nu-Chek Prep (Elysian, MN) with a purity higher than 98%. Deuterium labeled Arachidonic acid-d_8, (±)11,12 DHET-d_{11}, prostaglandin $F_{2\alpha}$-d_4, prostaglandin E_2-d_4, prostaglandin D_2-d_4, 6-keto prostaglandin $F_{1\alpha}$-d_4, 11(12) EET-d_{11}, 13(S)-HODE-d_4, 5(S)-HETE-d_8 Lipid Maps MS, 5(S), 6(R)-Lipoxin A_4-d_5, and (±)14,15DHET-d_{11} were used as internal standards for quantitation and were purchased from Cayman Chemical (Ann Arbor, MI) with purity higher than 99%. HPLC-grade acetonitrile (ACN), methanol (MeOH), and ethanol (EtOH) were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid (98% pure) was obtained from EMD Millipore (Billerica, MA). HPLC-grade hexane was purchased from Honeywell (Radnor, PA). Butylated hydroxytoluene (BHT) (99% pure) was obtained from Spectrum Chemical (New Brunswick, NJ). Eicosanoid common names, precursor masses (in *m*/*z*) and MS/MS fragments with retention times are listed in Supplemental Table 1.

2.2. Standard stock solutions

Each eicosanoid standard was individually prepared in a stock solution of ethanol at concentrations ranging from 1 μg/mL to 5 μg/mL, each with an addition of 500 μL 0.1% BHT (volume fraction in Milli-Q water). Internal standards were selected and assigned to each representative eicosanoid class monitored in this study for quantitation, as shown in Supplemental Table 1. The deuterium-labeled IS stock solutions were prepared at concentrations of 1 μg/mL–5 μg/mL in ethanol with an addition of 500 μL 0.1% BHT for quantitation. Stock solutions that contained mixtures of different eicosanoid species (not isobaric) were prepared in ethanol at a concentration of 1 μg/mL (with the addition of 500 μL 0.1% BHT) and were utilized (with IS added) for instrument tuning, LC method optimization and extraction method development. Calibration curves were constructed after an extraction method was developed for alligator CAM tissue (detailed further below).

2.3. LC-MS/MS method development and optimization

An AB Sciex API 4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer in negative electrospray ionization mode (ESI) was used for the analysis of eicosanoids in this study. Eicosanoid standards were analyzed for compound specific fragmentation patterns, which were examined at various collision energies until three to four candidate fragments were identified for each compound. Multiple reaction monitoring (MRM) was employed for each eicosanoid standard and after the optimal MRM transitions were identified (mass of precursor ion to a specific fragment ion), source-specific parameters were optimized for every eicosanoid standard using mixtures previously described, as shown in Supplemental Table 1. Curtain gas (CUR), cell exit potential (CXP), entrance potential (EP), nebulizer gas (GS1), heater gas (GS2), interface heater (ihe), ion spray voltage (IS), and source heater (TEM) settings were determined based on which settings were optimal across all eicosanoid measured (CUR = 10 psi, TEM = 525 °C, GS1 = 30 psi, GS2 = 30 psi, ihe =

on, CAD = High, and IS = 4500 V). Compound-specific settings, declustering potential (DP) and collision energy (CE), were optimized and employed for each eicosanoid. The optimized MRM transitions and compound specific MS settings are shown in Supplemental Table 1.

A Phenomenex Kinetex C18 column (100 mm × 2.1 mm, 2.6 μm particle size, Torrance, CA) was used with an Agilent 1200 series high performance liquid chromatography (HPLC) system equipped with a binary pump and autosampler. The column was maintained at 25 °C and the injection volume was set to 5 μL. The mobile phase gradient for the chromatographic separation of eicosanoids utilized two phases: (A) acetonitrile and (B) water with 0.1% formic acid (volume fraction in Milli-Q water). A flow rate of 250 μL/min was used to deliver the mobile phase using the following gradient scheme: 0–10 min 30% A, 12–14 min 90% A, 16–18 min 100% A, and 20–30 min 30% A. The addition of a 10 min equilibration step prior to the method was added to aid in improving reproducibility of chromatography and retention time. Scheduled MRM (sMRM, MRM transition employed at retention times specific for individual eicosanoids) was employed for detection of each eicosanoid in the developed LC-MS/MS method, with a 270 s detection window and a target scan time of 2 s.

2.4. Chorioallantoic membrane (CAM) sample collection and storage

2.4.1. Chicken (Gallus gallus)—Fertile chicken eggs were obtained from Pilgrim's Pride Corporation (Sumter, SC), incubated at 37 °C, and staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Six CAMs were collected from embryonic day (ED) 8, 10, 12, 14, 16, 18, and 20. The CAMs were dissected from the embryo and eggshell, rinsed three times in phosphate buffered solution, centrifuged to remove red blood cells, and flash frozen in liquid nitrogen. Chicken CAMs utilized for the COX assay were rinsed in Tris buffer (pH 7.4) and centrifuged at 10,000*g* for 10 min at 4 °C to separate red blood cells in accordance to the COX assay methodology (Cayman Chemical; Ann Arbor, MI).

All chicken CAM samples used for eicosanoid analysis were cryo-homogenized in liquid nitrogen with mortar and pestle and stored at −80 °C until LC-MS/MS analysis.

2.4.2. American alligator (Alligator mississippiensis)—Fieldwork was conducted under permits from the Florida Fish and Wildlife Conservation Commission (#SPGS-10-44). In 2013, 4 clutches of American alligator eggs were transported from Lake Woodruff National Wildlife Refuge (Deland, FL) to the Hollings Marine Laboratory for incubation. Upon arrival, eggs were candled for viability and 2–3 eggs were used to determine the average stage of embryonic development for each clutch based on the Ferguson staging guide (Ferguson, 1985). Approximately 10–14 eggs from each clutch were taken and distributed equally to either female producing temperature (30 °C) or male producing temperature (33 °C). At stages 19, 23 and 27, the CAM tissue was dissected from the embryos, rinsed three times in phosphate buffered solution (Cruze et al., 2013), centrifuged to remove red blood cells, and flash frozen in liquid nitrogen. These stages were utilized to investigate the changes in the eicosanoid milieu in CAM during three stages of middle and late development in the alligator (Ferguson and Joanen, 1982); alligator embryonic stages 19, 23, and 27 are equivalent to chicken embryonic days 10–12, 14–16, and 20, respectively.

At Stage 19 in the alligator embryo, distinct eyelids (upper and lower) are formed, which is similar to the chicken at ED 10 (Hamburger and Hamilton, 1951). Stage 27 in the alligator is distinguished by the egg yolk entering the abdominal cavity, which is mirrored at ED 20 in the chicken (Hamburger and Hamilton, 1951). Earlier stages were not collected given fieldwork constraints. From these samples, seven CAMs of each stage were cryo-homogenized by mortar and pestle with liquid nitrogen, extracted by solid phase extraction (SPE, method below), and analyzed using LC-MS/MS.

2.5. Extraction development with QC material and calibration curves

Quality control matrix-matched (QC) samples were prepared from a collection of 36 random American alligator chorioallantoic membrane tissue during embryonic development (between stages 10 and 28). The QC samples were cryo-homogenized with liquid nitrogen in a Retsch Cryomill (Newton, PA) equipped with a stainless steel 50 mL jar and grinding ball. QC material was created for the purpose of (1) extraction method development and determination of eicosanoids present in alligator CAM tissue, (2) investigating optimal storage conditions for the stability of eicosanoids in tissue, and (3) examining the reproducibility of the developed method over multiple days. As the eicosanoid milieu in CAM tissue for the alligator was unknown, it was important to investigate multiple extraction strategies to find the most appropriate method for this unique matrix. A QC material was also made using chicken CAM samples; a total of 32 random CAMs were collected during chicken development and homogenized/prepared using the same procedures detailed in the alligator QC material description. The chicken and alligator QC material was utilized in the development experiment to investigate the coefficient of variation (CV) in eicosanoid measurement from 5 replicate extractions.

Liquid-liquid (Maskrey and O'Donnell, 2008; Ostermann et al., 2014) and solid phase extraction (SPE) (Deems et al., 2007) were evaluated using different solvents (and solvent combinations; MeOH versus methyl formate) and standard spikes, and SPE was determined to be the preferred choice for further analysis due to the fact that liquid-liquid extraction was not favorable for the extraction of several eicosanoids, including the HODEs, HETEs, and PUFAs (data not shown). The alligator QC material was used to determine the amount of tissue optimal for sufficient eicosanoid response post-extraction and LC-MS/MS analysis and it was determined that approximately 150 mg of homogenized QC CAM tissue was required for reasonable detection of eicosanoids. After weighing of the tissue, with mass recorded, 1.5 mL of ice-cold 15% methanol was added to the sample. The sample was vortexed for 30 s and centrifuged at 4000*g* for 10 min at 4 °C. After centrifugation, the CAM tissue samples were acidified with 50 μL 0.1% formic acid (in Milli-Q water) and spiked with 50 ng of the internal standard mixture. Supelco SPE (Bellefonte, PA) LC-18 cartridges (6 mL) were used for the extraction procedure; prior to sample addition, the columns were conditioned with 5 mL methanol and 5 mL Milli-Q water. Immediately after acidification, the samples were loaded onto the SPE columns and were washed with 5 mL 15% methanol/85% Milli-Q water, 5 mL Milli-Q water, and 2.5 mL of hexane. The procedure was performed on a 12-port vacuum manifold (Supelco VisiPrep™), with a pressure that did not exceed 5 Hg. After the washing step, the samples were then eluted with 5 mL of methanol, dried under a gentle stream of nitrogen, and reconstituted in 100 μL of the mobile

phase (30% ACN/70% water). An extraction efficiency experiment was performed to determine the optimal elution phase for spiked eicosanoids in CAM tissue; methanol resulted in a 20–30% better recovery of PUFAs (AA, DHA, DPA, and EPA), 11,12-EET, 9-HODE and 5-HETE in comparison to the commonly used solvent methyl formate (Le Faouder et al., 2013) (data not shown). Variation in the reproducibility of the 6 QC replicates was also decreased in all eicosanoids analyzed using the eluent methanol versus methyl formate.

After extraction, calibration curves for each eicosanoid present in the QC samples were made at concentrations that ranged from 0.01 ng/mL to 10000 ng/mL in EtOH; calibration lines were calculated using the linear regression method of least squares. All curves had an R^2 above 0.97, with specific equations for each analyte measured shown in Supplemental Table 2. The limit of detection (LOD) for the each experiment was calculated based on mean peak area of method blanks plus 3 times the standard deviation of the noise adjusted to the mean blank with internal standards. Ethanol and 70%/30% ACN/Milli-Q water blanks were both analyzed and had no difference. LODs for the alligator and chicken experiment are shown in Supplemental Tables 2 and 3.

2.6. CAM eicosanoid measurement and statistical analysis

For the alligator CAM developmental experiment, a total of 32 samples were analyzed in two sample batches: with 5 QC material samples, n = 7 CAM samples per stage, and 6 method blanks. A total of 18 calibration curves were constructed for each eicosanoid measured, from the previous QC extraction experiments, and analyzed with the samples. Analytes from both batches were adjusted to the appropriate internal standard and normalized to the tissue wet-mass.

The chicken development experiment analyzed a total of 44 samples in two batches: with 4 QC material samples, n = 3–4 samples per embryonic day, and 12 method blanks. A total of 10 calibration curves were constructed and analyzed with these samples. For analysis, individual samples from each embryonic day (8–20) were grouped into “Early development – ED” (Stage 8 and 10, n = 10), “Mid-development – MD” (Stage 12 and 14, n = 9), and “Late Development – LD” (Stage 16, 18, and 20, n = 9).

The QC materials from both alligator and chicken were also analyzed for reproducibility and data quality during the developmental experiment. Eicosanoid intra- and inter-assay variation was less than 12%, with the exception of DHA, 14,15-EET, 14,15-DHET, and 11,12-DHET (Supplemental Tables 2 and 3). The reproducibility of these compounds in alligator QC tissue was not optimal, and further analysis into their stability in this tissue is warranted. Final measurements of each eicosanoid were calculated from calibration curves, normalized to wet tissue weights, and the concentrations were expressed as ng eicosanoid/g wet weight (or pg/g wet weight depending on the eicosanoid). Peak integration was performed using Analyst Software v1.5.2 and statistical analysis (Kruskal-Wallis test, One-way ANOVA) was performed utilizing GraphPad Prism 6.0 and significance was determined using $p < 0.05$.

2.7. Cyclooxygenase (COX) assay

To further investigate the biosynthesis of eicosanoids in the chicken CAM tissue, we examined the activity of total cyclooxygenase activity in the chicken CAM at embryonic days 8, 12 and 18. Enzymatic activity was measured using a COX activity assay kit (Cayman Chemical, Ann Arbor, MI). The kit used N,N,N,N-tetramethyl-*p*-phenylenediamine (TMPD) as a colorimetric substrate to measure peroxidase activity, the second mechanism of the COX pathway. Six samples from ED 8, 12, and 18 were assayed for COX activity according to the kit manufacturer's suggested protocol.

3. Results

3.1. Developmental changes of eicosanoids in alligator CAM tissue and reproducibility of method

All PUFA precursors of the eicosanoid pathway increased over the three sampled stages of alligator development. Arachidonic acid had the greatest increase between stage 19 and stage 27 ($p < 0.0001$), followed by DHA ($p = 0.02$), DPA ($p = 0.0256$), and EPA ($p = 0.0316$), as shown in Fig. 2a. There were no changes found between stages 19 and 23 in the four PUFAs measured in CAM tissue.

Cyclooxygenase (COX) derived metabolites, with the exception of 6-Keto-$PGF_{2\alpha}$, increased throughout development in CAM tissue (Fig. 2b). PGE_2 ($p = 0.0003$) and 11-HETE ($p = 0.0006$) presented the strongest changes during development in alligator CAM between stages 19 and 27. PGD_2 ($p = 0.0026$), TXB_2 ($p = 0.0032$) and $PGF_{2\alpha}$ ($p = 0.0175$) also had differences between stages 19 and 27. 6-Keto-$PGF_{2\alpha}$, on the other hand, had a decrease during embryonic development in the alligator CAM – between stages 19 and 27 ($p = 0.0331$).

15-HETE and 5-HETE were detected in the alligator CAM tissue. 15-HETE ($p = 0.0052$) had an increase in concentration in the CAM tissue from stage 19 to stage 27, as shown in Fig. 2c. There was no statistical difference between stage 19 and stage 23 for 15-HETE. 5-HETE exhibited no statistical difference between all three sampled stages of alligator development. 11,12-EET had an increase in CAM tissue from Stage 19 to Stage 27 ($p = 0.0004$); 14,15-EET also had an increase from stage 19 ($p = 0.0035$). Both did not have an increase from stage 19 to stage 23. 14,15-DHET and 11,12-DHET showed no differences throughout embryonic development. Two hydroxyoctadecadienoic acids were examined in the alligator CAM tissue (13-HODE and 9-HODE). 13-HODE had an increase between stage 19 and 27 ($p = 0.0367$), and 9-HODE had the same result ($p = 0.045$). However, both did not exhibit a difference between stages 19 and 23 in the alligator CAM.

In the QC material, all eicosanoids examined in the method had a coefficient of variation (CV) below 15%, with the exception of DHA, 11,12-DHET, 14,15-DHET, and 14,15-EET (shown in Supplemental Tables 2 and 3). Both DHETs and 14,15-EET were measured at levels close to the LOD; which might account for the variation in the QC material.

3.2. Developmental changes of eicosanoids in chicken CAM tissue and QC reproducibility

In this aspect of the study, we measured eicosanoid precursors (PUFAs) and COX-derived metabolite concentrations in the chicken CAM during embryonic development and compared those changes to our observations in alligator CAM.

Four PUFAs decreased between ED and LD. AA and DHA had the strongest change between ED and LD ($p < 0.0001$), followed DPA ($p = 0.0001$) and EPA ($p = 0.004$), as shown in Fig. 3a. As with the alligator CAM, there were no changes found between ED and MD in the four PUFAs measured in chicken CAM tissue. Of note, all PUFAs were measured at ng/g wet weight levels – a marked decrease from the μg/g wet weight concentrations measured in alligator CAM.

Consistent with the alligator CAM results, cyclooxygenase (COX) derived metabolites showed developmental increases in chicken CAM tissue (Fig. 3b). 11-HETE ($p < 0.0001$) presented the strongest increase in concentration during development in chicken CAM between ED and LD, shown in Fig. 1. PGE_2 ($p = 0.0188$), PGD_2 ($p = 0.0002$) and $PGF_{2\alpha}$ ($p = 0.0001$) also had differences between ED and LD of chicken development in chicken CAM tissue. TXB_2 and 6-Keto-$PGF_{2\alpha}$ were below the limit of detection for chicken CAM samples and are not shown in Fig. 3.

3.3. COX activity in chicken CAM tissue

To compliment results from the LC-MS/MS assay, we examined total cyclooxygenase activity in the chicken CAM at embryonic day 8, 12 and 18 ($n = 6$, each day). An increase ($p < 0.0001$) in total COX activity was observed between day 8 and 18 as shown in Fig. 4. The COX results observed in the chicken CAM are consistent with the increased PGE_2, $PGF_{2\alpha}$, and 11-HETE detected in the CAM during embryonic development, as shown in the eicosanoid pathway Fig. 1.

4. Discussion and conclusions

Within the last 10 years, numerous technical advances in both human and model organism studies have produced several high-throughput ‘omics’ based assessments of eicosanoids both *in vitro* and *in vivo* (Buczynski, 2009; Dumlao et al., 2012; Norling and Serhan, 2010; Serhan, 2009). While this trend has gained traction in the biomedical sciences community, it has recently surged in the environmental monitoring and comparative endocrinology arena (Garcia-Reyero et al., 2014; Jungnickel et al., 2014; Scanlan et al., 2015; Schneider and Orchard, 2011). In this study, we created a new ‘eicosanomic’ strategy for the quantitation of the eicosanoid milieu in non-traditional species, models, and/or matrices. Here, we demonstrated this strategy by examining the developmental differences in CAM tissue between two oviparous species. This is the first investigation of the eicosanome in the CAM tissue of an oviparous reptile and marks an important comparative assessment of oviparous species.

Traditionally, radioimmunoassays (RIA) and enzyme immunoassays (EIA) have been used to measure specific eicosanoids in plasma, tissue and urine (Anhut et al., 1978; Demers and Derck, 1980; Maclouf et al., 1986; Reiss et al., 1997; Tae-Rim Shin et al., 2015). While

these assays are robust, they measure compounds one-at-a time and can be troublesome for nontraditional species due to issues with the lack of commercially available antibodies. The concept of measuring multiple pathways within the eicosanoid family using LC-MS/MS is useful for establishing trends or highlighting specific pathways of interest (Blaho et al., 2009; Capra et al., 2014). Further, the advantage of using LC-MS/MS for the measurement of the eicosanome in nontraditional species is that it does not require antibodies to measure, nor is dependent on the sample matrix – the technique utilizes examination of the chemical structure to identify and quantify specific eicosanoids in tissue and other biological matrices. The accuracy and precision of the method and each experiment was assessed using an in-house QC material, an approach not typically employed for measurement of eicosanoids in biological tissue. Though we did not have below 15% variation in all metabolites assessed, as shown in Supplemental Tables 2 and 3, we did use the information gained from this experiment to highlight specific aspects in need of improvement, such as sample storage and handling procedures for eicosanoids with high variation (DHETs, DHA). Furthermore, this is one of the first investigations to use a QC material to assess eicosanoid changes due to extraction and variability of the LC-MS/MS methodology. In our examination of both the chicken and alligator QC material, we found that several classes of eicosanoids (14,15-EET and DHETs) had low reproducibility and hypothesize that this is due to the trace levels of these compounds present in the CAM tissue. We highlight the need to create an in-house reference material for measurement of eicosanoids in each non-model species or matrix due to the uncertain stability of eicosanoids, and also advise using this material to routinely assess the reproducibility of both the extraction and LC-MS/MS methodology during measurement.

All amniotes, viviparous and oviparous, develop identical extraembryonic membranes and thus, examination of the eicosanome in the chorioallantoic membrane of these species could potentially provide a framework to ascertain evolutionary conserved characteristics. Previously, Cruze et al. found steroidogenic activity in three oviparous species and shifted the understanding that steroid activity and metabolism was a feature found only in mammalian placental amniotes (Albergotti et al., 2009; Cruze et al., 2012). Cruze et al. proposed that endocrine activity is evolutionary conserved across all amniotes (Cruze et al., 2013), and did not arise with the evolution of vivaparity. We further support this hypothesis with the first evidence of ‘local’ hormones (eicosanoids) in the CAM tissue of an oviparous reptile. We also investigated eicosanoid levels and cyclooxygenase activity in the chicken CAM tissue and found similar trends during embryonic development. To summarize, both the alligator and chicken CAM had increases in several COX-derived metabolites of the eicosanoid family (PGE_2, 11-HETE, $PGF_{2\alpha}$), as shown in Figs. 1 and 2. The two species differed in that the alligator CAM had increased PUFA concentrations during development, while the chicken PUFAs decreased throughout embryonic development.

The prostaglandin (PG) family has been extensively investigated in mammalian placental development and reproduction. In sheep placental cells, low levels of PGs were measured in early embryonic development and results showed a marked increase in PG production after 100 days of gestation (Challis et al., 1997; Kelly, 1996; Liggins et al., 2008). Subsequently, measurement of circulating PGE_2 in the uterine vein of the sheep showed a large increase during the last third of gestation (Challis et al., 2002; Thorburn, 1991), indicating that a

large amount of PGE_2 was synthesized prior to parturition in the mammalian placenta. Increased PGE_2 during late embryonic development in the placenta has been observed in pigs, rabbits, and humans (Franczak et al., 2006; Kelly et al., 2009; Kubota et al., 2005; Murakami et al., 2002; Snegovskikh et al., 2006). To review, low levels of prostaglandins are typically found in the placenta prior to a large increase in the last third of gestation across a variety of mammalian species. In both the chicken and alligator CAM, the PG pathway showed the highest level of change during embryonic development. As shown in Figs. 2 and 3, PGE_2, AA, and 11-HETE had the largest increase from early development to the latest stage of development sampled in both the chicken and alligator CAM tissue (alligator between stage 19 and stage 27; chicken between ED and LD). Interestingly enough, AA and other PUFAs increased in the alligator CAM during development, while the chicken PUFAs decreased. One would expect the decrease given that more of the 'product' from the COX pathway was observed in the later stages of development (shown in Fig. 1B); however, it has been shown in humans that free fatty acids (including AA, and DHA) increase dramatically during the third trimester in the placenta (Okita et al., 1982; Parisi and Walsh, 1986; Wang et al., 2006). A future investigation into eicosanoid measurement of other extraembryonic membranes (yolk sac membrane) in both species is needed to determine if the chicken and alligator differ in how PUFAs are mobilized to the embryo during embryonic development. One hypothesis for the difference observed between the alligator and chicken is that they preferentially deposit/release PUFAs from different sources in the eggshell (yolk sac membrane vs chorioallantoic membrane); however, those conclusions cannot be made from this study.

Further work has shown that there is a large increase in cyclooxygenase (COX) activity in the placenta between early embryonic development and late embryonic development (Matsubara et al., 1997; Wetzka et al., 1997) which helps corroborate the increased PG production. This increased COX activity has been observed in a variety of species as well (Loftin et al., 2002; Xu et al., 2005), and alterations in this pathway can lead to detrimental effects for the embryo (i.e. respiratory problems, lung maturation, preeclampsia) (Alvaro et al., 2004; Boone et al., 1993; Friedman, 1988; Olofsson and Leung, 1994; Zhang et al., 2014). We showed a similar increasing trend of COX activity in the chicken CAM during the last third (LD) of embryonic development, and hypothesize given the large amounts of PGs in the alligator CAM that it would show a similar increase during later development. To our knowledge, this is the first examination of endogenous COX activity during embryonic development in the CAM tissue of an oviparous species. There have been numerous investigations of COX activity in the CAM following treatment with potential angiogenic pharmaceuticals; the chicken CAM is used to test many pharmaceuticals and other agents for angiogenic activity (Blacher et al., 2005; Nowak-Sliwinska et al., 2014; Ribatti, 2012; Valdes et al., 2002); however, to the authors knowledge, no study exists which investigates the ontogeny of COX activity during development, nor examines the endogenous levels of PG and other lipid mediators without alteration through treatment with exogenous pharmaceuticals. In this realm, it would be prudent to investigate the endogenous role of these mediators prior to treatment or exposure to further assess the natural variation in these biological tissues. We must first investigate the normal biological processes during

development to understand if abnormalities are attributed to exposure or are part of the natural variation in biological systems (Guillette and Guillette, 1996; Guillette, 2006).

Prostaglandins and other lipid mediators have been assessed in comparative species using a variety of techniques; notably, Guillette et al. established prostaglandin synthesis in the liver and oviduct of a reptilian species (*Sceloporus jarrovi*) (Guillette et al., 1988, 1990). Guillette et al. also saw increased levels of $PGF_{2\alpha}$ in circulation prior to egg-laying in the sea turtle (*Carreta carreta*) (Guillette et al., 1991). However, to date, few studies have been performed examining oviparous reptiles with regards to PG and other lipid mediator synthesis and regulation during embryonic development. Notably, other pathways in the eicosanoid family, such as lipoxygenases or epoxyeicosatrienoic acids, have not been well characterized in the reptilian CAM and this study is one of the first few to investigate the presence of EETs, HETEs and HODE metabolites in this tissue during embryonic development. Though the PG pathway and 11-HETE (COX-derived products – Fig. 1) showed the highest increase during the late period of development in the alligator CAM, other pathways besides the classical COX were also increased. 11,12-EET and 15-HETE also had an increase in the later stage of development for the alligator CAM. Studies of lipoxygenase metabolites (15-HETE) during pregnancy in the mammalian placenta have also been shown to have an increase prior to parturition (Morrison et al., 1984); alterations (or increased levels of 15-HETE) in the placenta has been attributed to oxidative stress and correlated with preeclampsia (Johnson et al., 1998; Mitchell and Grzyboski, 1987). These investigations highlight the need for comparative investigations into lipoxygenase metabolites, as well, during embryonic development. Further assessments of oxidative stress to the embryo are important for supplementing our knowledge of placental pathologies, such as preeclampsia and pre-term birth.

Unlike previous reports in mammalian placenta (Schaefer et al., 1997), 11,12-DHET and 14,15-DHET did not change with respect to embryonic development in the alligator CAM. Oxidized products of linoleic acid (13-HODE and 9-HODE) did increase with development in the alligator CAM; however, levels of these epoxyeicosatrienoic acids are unknown within the mammalian placenta during pregnancy – though it is known that the placenta has the required enzymatic machinery to secrete these oxidized lipids (Fang et al., 1999; Fournier et al., 2011).

All viviparous amniotes share a similar characteristic of increased PGE_2 and $PGF_{2\alpha}$ levels prior to parturition (Olson, 2003). In both the avian and reptilian CAM, several eicosanoids presented here are increased in concentration prior to hatching. The COX-derived PGs are the highest in concentration of all the eicosanoid metabolites in this tissue for both chicken and alligator. We hypothesize that during development of the CAM tissue, increased concentrations of COX-derived mediators are essential to aid with increased vascularization and respiration in this tissue before hatching, as many of these lipid mediators are required for angiogenesis, vascularization, and maintenance of blood flow (Form and Auerbach, 1983; Gronert et al., 1999; Hoggatt and Pelus, 2010; Kawahara et al., 2015; Von Aulock et al., 2003). Though we did not explore the bioactivity of these compounds in the CAM tissue, we have characterized the eicosanoid composition in the reptilian CAM and examined their change with respect to embryonic development. Further, we compared reptilian COX-

derived mediators and PUFAs to the avian CAM and examined COX activity in this tissue. Increased COX activity in the chicken CAM supports the evidence that this tissue has the required machinery to synthesize eicosanoid products from PUFAs. Future investigations into PG synthesis in the alligator CAM is needed to determine whether PGs and other lipid mediators are synthesized in this tissue; however, given that the chicken CAM is capable of COX activity, we hypothesize that the reptilian CAM shares similar activity of COX given the high amounts of PGE_2 measured prior to hatching. Future directions will investigate the biological activity of PGE_2 and $PGF_{2\alpha}$ in the reptilian CAM, and investigate if altering these lipids in the CAM has detrimental effects to the embryo. Specifically, investigating these compounds during reproduction and embryonic development can help shed insight into how these lipid mediators are evolutionarily conserved during these processes and their potential role in immune function within the CAM. Furthermore, we can utilize the CAM to investigate abiotic factors in the environment (e.g., temperature, contaminants) and how they affect eicosanoid biosynthesis within this tissue during development.

5. Disclaimer

Certain commercial equipment or instruments are identified in the paper to specify adequately the experimental procedures. Such identification does not imply recommendations or endorsement by the NIST nor does it imply that the equipment or instruments are the best available for the purpose.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.07.006.

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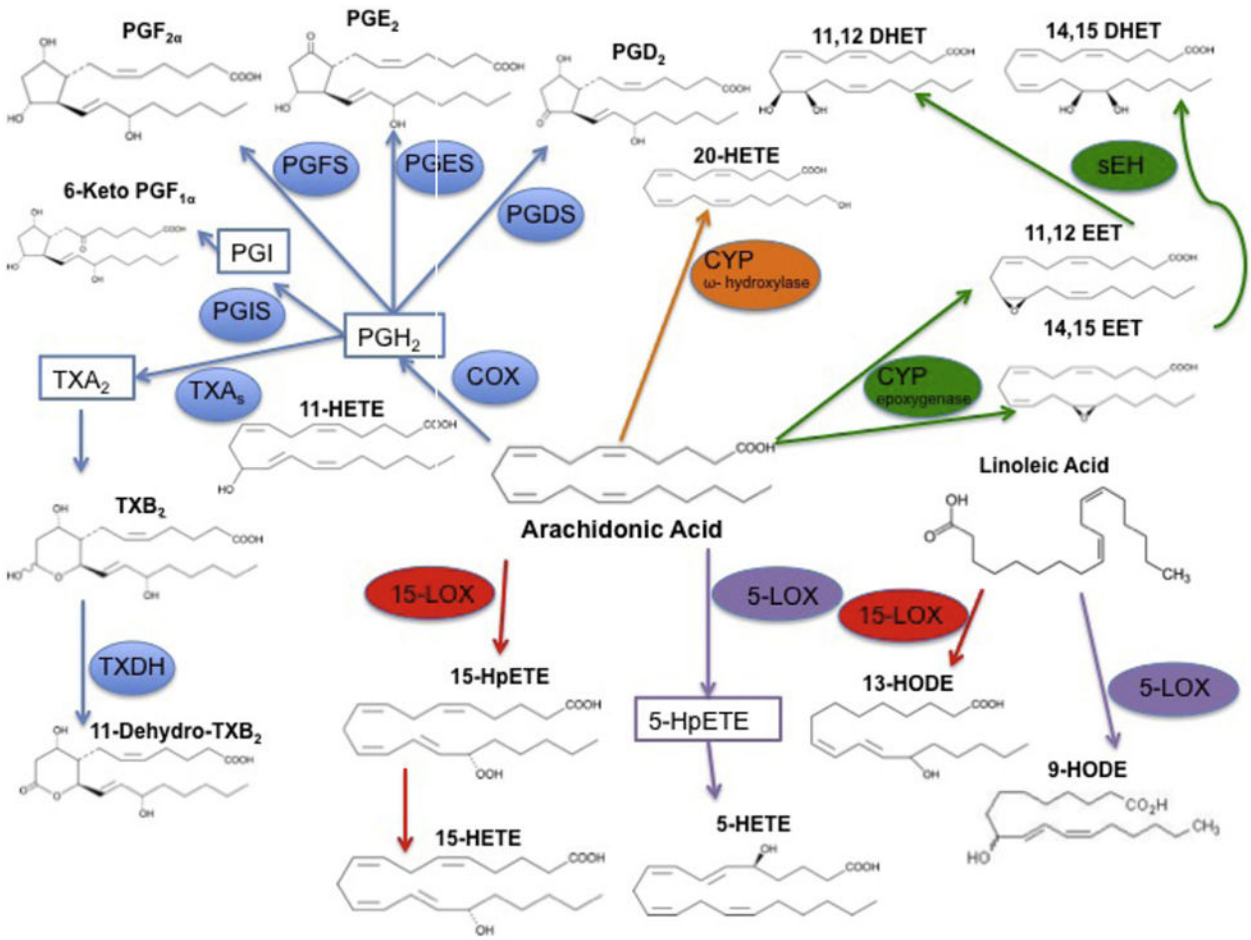

Fig. 1.

Representative arachidonic acid pathway of eicosanoid metabolism with common names and structures. Enzymatic pathways are highlighted in different colors, enzymes are encircled, and intermediates are labeled with the common name.

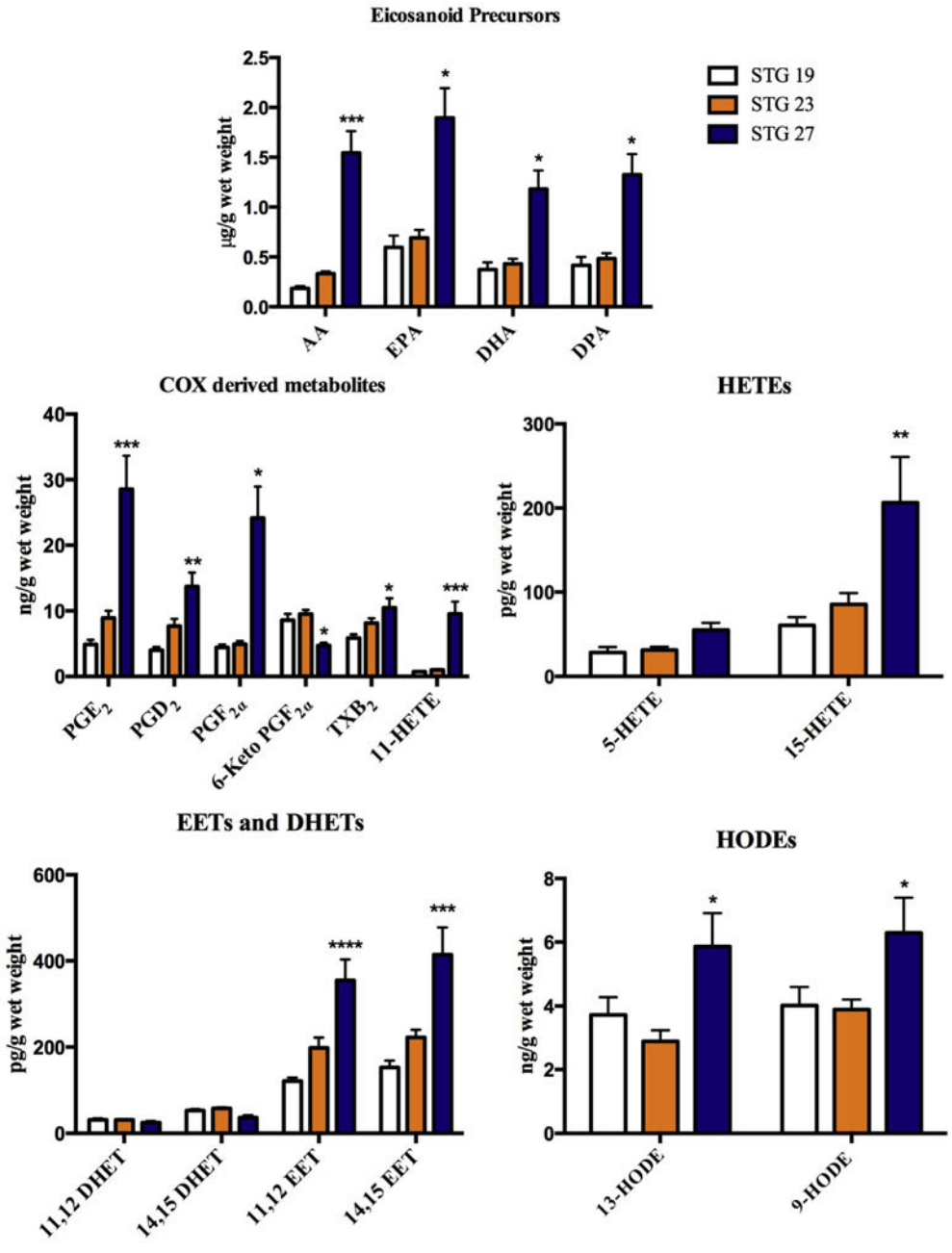

Fig. 2.

(A) Measured quantities (Mean ± SEM) of AA, EPA, DHA, and DPA in alligator CAM tissue expressed as µg/g wet mass. (B) Measured quantities of PGE_2, PGD_2, PGF_{2a}, 6-Keto-PGF_{2a}, TXB_2, and 11-HETE in alligator CAM tissue expressed as ng/g wet weight. (C) Measured quantities of 15-HETE and 5-HETE in alligator CAM tissue expressed as pg/g wet weight. (D) Measured quantities of 11,12-DHET, 14,15-DHET, 11,12-EET, and 14,15-EET in alligator CAM tissue expressed as pg/g wet weight. (E) Measured quantities of 13-HODE and 9-HODE in alligator CAM tissue expressed as ng/g wet weight. n = 7 per stage for all measurands, Kruskal-Wallis test, $^{****}p < 0.0001$, $^{***}p < 0.0003$, $^{**}p < 0.01$, $^{*}p < 0.05$.

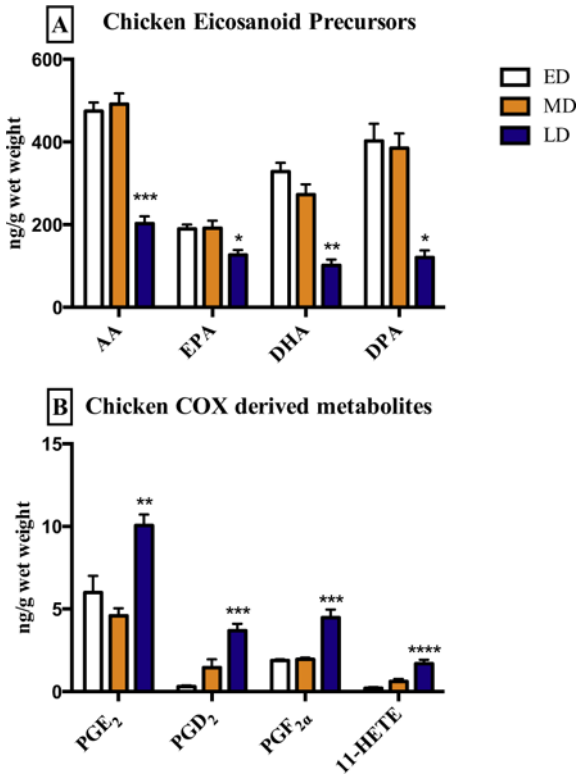

Fig. 3.

(A) Measured quantities (Mean ± SEM) of AA, EPA, DHA, and DPA in chicken CAM tissue expressed as ng/g wet weight. (B) Measured quantities of PGE_2, PGD_2, PGF_{2a}, and 11-HETE in chicken CAM tissue expressed as ng/g wet weight. ED, early development (embryonic days 8 and 10), n = 10; MD, mid-development (embryonic days 12 and 14), n = 9; LD, late development, n = 9 (embryonic days 16, 18, and 20). Kruskal-Wallis test, One-way ANOVA. ****$p < 0.0001$, ***$p < 0.0003$, **$p < 0.01$, *$p < 0.05$.

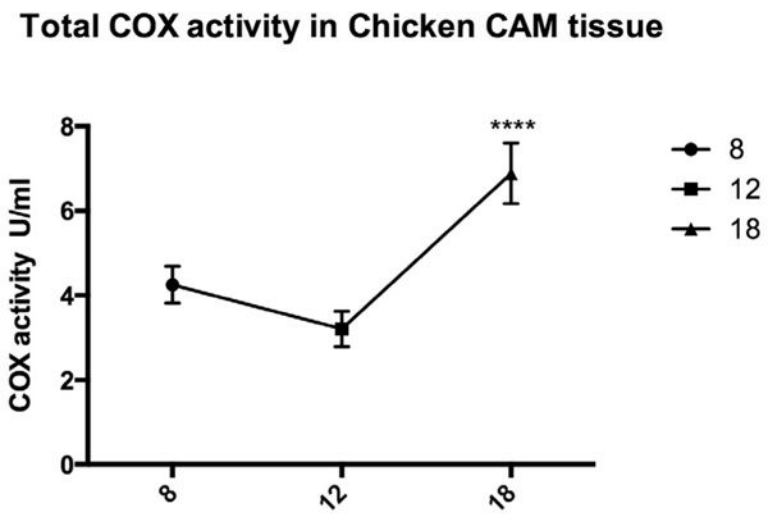

Fig. 4.

Measured quantities (Mean ± SEM) of total COX activity (U/mL = nmol/min/μL) chicken CAM tissue. 8, 12 and 18 correspond to days of embryonic development; Day 8 (n = 6), Day 12 (n = 6), Day 18 (n = 6). Kruskal-Wallis test, One-way ANOVA. $^{****}p < 0.0001$.