THE EVOLUTION OF ENDOCRINE EXTRAEMBRYONIC MEMBRANES; A COMPARATIVE STUDY OF STEROIDOGENESIS AND STEROID SIGNALING IN THE CHORIOALLANTOIC MEMBRANE OF OVIPAROUS AMNIOTES

By

LORI CRUZE ALBERGOTTI

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2011

Ken, I will be forever grateful for your love and support.

ACKNOWLEDGMENTS

First, I would like to thank Lou Guillette for his generous financial, technical and emotional support. Lou has taught me that being a scientist is the opportunity to be a detective in pursuit of answers to an intriguing question, and this pursuit requires not only an analytical mind, but also a creative one. I thank Lou for giving me this opportunity and for his endless guidance and encouragement, and last but not least for his friendship. I could not have asked for a better graduate experience and I am convinced that this has everything to do with having Lou as an advisor and Bernie, Colette, Marty and Malcolm as committee members. I would like to thank Bernie for pushing me outside of my comfort zone, for encouraging me to think broadly about my research, and for his willingness to provide lab assistance. Thank you to Colette for lending statistical and experimental design expertise, for pushing me to think about the big picture of my research, and for always believing in me. I would like to thank Marty for expanding my understanding of evolution and development, for allowing me the amazing opportunity to teach with him, and for making his lab available to me. Thank you to Malcolm for his expertise in developmental signaling, for his constant enthusiasm, and for his willingness to the take time to hear how things are going and offer words of encouragement.

I would also like to thank the Department of Biology for providing a wonderful academic home for the last five years. I would like to thank the Biology faculty in general for being excellent academic role models and in particular I would like to thank Rebecca Kimball, Ed Braun, Charlie Baer, Marta Wayne and Dave Evans for taking an interest in me, offering guidance and assistance, and for helping me become a better scientist. I would also like to thank Keith Choe for the generous use of his microscope and camera.

Thank you to my lab mates, colleagues and friends from whom I have learned so much. Thanks to Satomi Kohno and Brandon Moore for teaching me molecular biology, for providing technical advice, and for helping me hit the ground running. Thank you to Thea Edwards and Alison Roark for sharing my interest in the placenta and extraembryonic membranes and for the countless hours of conversation that helped to define my ideas about the evolution of these structures. Thank you to Heather Hamlin for her assistance with tissue culture and hormone techniques, for her boundless energy and amazing spirit. Thank you to Krista McCoy, Leslie Babonis, Kelly Hyndman, and Marda Jorgensen for providing histology, immunohistochemistry and microscopy training and advice. Thank you to Mike McCoy for providing statistical expertise and support and for pushing me to think outside the box about data analysis. Thank you to Ashley Boggs and Nicole Botteri for sharing this experience with me and for their continuous support and friendship. Thanks to the wonderful undergraduate students that I have had the pleasure to mentor and without whose toiling hours in the lab this work would have been incredibly difficult; Chris Olmo, Yao Fu, Sasha Strul, Jacob Fyda, Shellah Palmer, Jenna Harty, Momna Younas, Gabrielle Rolland, and Patpilai Kasinpila. I would also like to thank the Florida Fish and Wildlife Conservation Commission for their assistance in the collection of alligator and turtle eggs.

Finally, I would like to thank my mom and dad for their love and encouragement. A special thanks to all of my friends, but especially Chip, Michelle, Jon, Christine, Stephanie and the EC, for keeping me sane these last few years. I thank my wonderful husband for making me laugh every day, for reminding me that life is a balance and for his willingness to join in this adventure with me.

TABLE OF CONTENTS

page

ACKNOWLEDGMENTS 4

LIST OF TABLES 9

LIST OF FIGURES 10

LIST OF ABBREVIATIONS 12

ABSTRACT 16

CHAPTER

1 INTRODUCTION 19

The Evolution of Viviparity 20
Egg Retention and the Lengthening of Gestation 21
The Reduction of the Eggshell 24
The Emergence of the Placenta 27
An Endocrine Placenta 30
A New Hypothesis for the Evolution of Endocrine Extraembryonic Membranes and the Placenta: Implications for the Evolution of Viviparity 32
Specific Hypotheses 33
Significance of Work 34

2 STEROIDOGENESIS AND STEROID SIGNALING IN THE CHORIOALLANTOIC MEMBRANE OF THE CHICKEN (*GALLUS GALLUS*) 37

Materials and Methods 39
CAM Collection 39
RNA Isolation and Reverse Transcription 39
Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) 39
Cloning and Sequencing of Plasmids 41
RT–qPCR Primers 41
Sexing of Embryos 41
In Vitro Explant Culture 42
Immunohistochemistry and Microscopy 42
Statistical Analysis 43
Results 45
The Chicken CAM has the Required Molecular Mechanisms to Perform Steroidogenesis and Synthesis of Progesterone 45
The Chicken CAM is Capable of *In Vitro* Progesterone Synthesis 46
The Chicken CAM is Capable of Responding to P4 Signaling Through the Progesterone Receptor 47

Discussion 49

3 THE CHORIOALLANTOIC MEMBRANE OF THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*) HAS THE CAPABILITY TO PERFORM STEROID BIOSYNTHESIS AND RESPOND TO STEROID HORMONE SIGNALING 56

Materials and Methods 60
Egg Collection and Sample Preparation 60
RNA Isolation and Reverse Transcription 61
Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) 61
RT-qPCR Normalization 62
Cloning and Sequencing of Plasmids 63
Immunohistochemistry and Microscopy 63
Statistical Analysis 64
Results 66
Internal Control Expression among Embryonic Stages and between Incubation Temperatures 66
Steroidogenic Factor, Enzyme and Steroid Receptor Expression among Embryonic Stages 67
Steroidogenic Factor, Enzyme and Steroid Receptor Expression between Incubation Temperatures 68
PR and ESR1 Immunolocalization 69
Discussion 69

4 THE OVIPAROUS CHORIOALLANTOIC MEMBRANE OF THE RED-BELLY SLIDER TURTLE (*PSEUDEMYS NELSONI*) HAS THE CAPABILITY TO SYNTHESIZE PROGESTERONE AND RESPOND TO STEROID HORMONE SIGNALING 94

Materials and Methods 97
Egg Collection 97
RNA Isolation and Reverse Transcription 98
Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) 98
Cloning and Sequencing of Plasmids 99
RT–qPCR Primers 100
In Vitro Explant Culture 100
Histology, Immunohistochemistry and Microscopy 101
Statistical Analysis 102
Results 103
The Turtle CAM is Capable of *In Vitro* Progesterone Synthesis 103
The Turtle CAM is Capable of Responding to Steroid Hormone Signaling 103
Discussion 104

5 ESTROGENIC EXPOSURE AFFECTS CHORIOALLANTOIC MEMBRANE GENE EXPRESSION AND TIMING OF HATCH IN THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*) 120

Materials and Methods 125
Egg Collection 125
In Ovo Exposure 125
CAM Collection, RNA Isolation, Reverse Transcription and Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) 126
Hatch Data 127
Statistical Analysis 127
Results 128
Steroid Receptor and Steroidogenic Enzyme Expression in the CAM Following Estrogenic Exposure 128
The Effect of Estrogenic Exposure on Timing of Hatch 128
Hatchling Body Morphometrics Following Estrogenic Exposure 129
Discussion 129

6 SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS 146

Summary and Conclusions 146
Future Directions 150

LIST OF REFERENCES 154

BIOGRAPHICAL SKETCH 179

LIST OF TABLES

Table		page
2-1	Chicken PCR primers used for RT-Quantitative real-time PCR	51
3-1	Alligator PCR primers used for RT-Quantitative real-time PCR	80
3-2	Statistic summary of RT-Quantitative real-time PCR on the alligator CAM at three embryonic stages	81
3-3	Statistic summary of RT-Quantitative real-time PCR on the alligator CAM at two incubation temperatures	82
4-1	Turtle PCR primers used for RT-Quantitative real-time PCR	113
5-1	Alligator PCR primers used for RT-Quantitative real-time PCR	137
5-2	Statistic summary of RT-Quantitative real-time PCR on the alligator CAM at four time periods post exposure	138

LIST OF FIGURES

Figure		page
1-1	The extraembryonic membranes of oviparous and viviparous squamates	35
1-2	General overview of the steroid hormone biosynthetic pathway	36
2-1	A simplified version of steroid biosynthesis highlighting the specific steroidogenic enzymes investigated	52
2-2	Relative mRNA expression of steroidogenic enzymes in the chick CAM.	53
2-3	Progesterone synthesis in the chick CAM	54
2-4	PR and ESR1 mRNA expression and PR immunolocalization in the chick CAM	55
3-1	mRNA expression of internal control genes in alligator CAM	83
3-2	Relative mRNA expression of steroidogenic factor and enzymes in the alligator CAM incubated at the FPT	84
3-3	Relative mRNA expression of steroidogenic factor and enzymes in the alligator CAM incubated at the MPT	85
3-4	Relative mRNA expression of steroid receptors in the alligator CAM incubated at the FPT	87
3-5	Relative mRNA expression of steroid receptors in the alligator CAM incubated at the MPT	89
3-6	Relative mRNA expression of NR5A1, HSD3B1, PR, and ESR2 in the alligator CAM between incubation temperatures at embryonic stage 19	90
3-7	Relative mRNA expression of GR and ESR1 in the alligator CAM between incubation temperatures at embryonic stage 23	91
3-8	PR immunolocalization in the alligator CAM	92
3-9	ESR1 immunolocalization in the alligator CAM	93
4-1	Progesterone synthesis in the turtle CAM	114
4-2	Relative mRNA expression of steroid receptors in the turtle CAM	115
4-3	Histology and immunolocalization of PR in turtle CAM	116

4-4 Amniote reproductive modes and evidence of steroid hormone synthesis and/or steroid signaling in extraembryonic membranes and placentae 117

4-5 A simplified version of progesterone biosynthesis 118

4-6 Model for the evolution of endocrine function in the amniote yolk sac (YSM) and chorioallantoic membrane (CAM) 119

5-1 Relative mRNA expression of ESR1 in the CAM following estrogenic exposure 139

5-2 Relative mRNA expression of ESR2 in the CAM following estrogenic exposure 140

5-3 Relative mRNA expression of PR in the CAM following estrogenic exposure .. 141

5-4 Relative mRNA expression of CYP19A1 in the CAM following estrogenic exposure 142

5-5 Relative mRNA expression of HSD3B1 in the CAM following estrogenic exposure 143

5-6 The effect of estrogenic exposure on timing of hatch 144

5-7 The effect of estrogenic exposure on hatchling body morphometrics 145

6-1 Evidence of endocrine extraembryonic membranes in amniotes 153

LIST OF ABBREVIATIONS

A	Androstenedione. An androgenic steroid that can act as a precursor in the synthesis of both estrogens and androgens.
ACTB	Actin, beta. A highly conserved cytoskeletal protein that is commonly used as an internal control gene in mRNA expression studies.
AR	Androgen receptor. A nuclear steroid receptor that is activated by binding of androgens.
CAM	Chorioallantoic membrane. An extraembryonic membrane composed of chorion and allantois.
cDNA	Complimentary DNA. DNA that is complementary to a certain sequence of messenger RNA template.
CL	Corpus luteum. A transitory endocrine organ formed from the post-ovulatory follicle.
CORT	Corticosterone. One of the glucocorticoid hormones.
CYP11A1	Cytochrome P450 11A1, also called cytochrome side-chain cleaving enzyme. Steroidogenic enzyme that converts cholesterol to pregnenolone.
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1, also called 17α-hydroxylase. Steroidogenic enzyme that is a key enzyme in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens, and estrogens.
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1, also called aromatase. Steroidogenic enzyme that converts androstenedione or testosterone to estrone or estradiol 17β, respectively.
DNA	Deoxyribonucleic acid. The genetic code required by all life to develop and function.
dNTPs	Deoxyribonucleotide triphosphate. Generic term referring to the four deoxyribonucleotides: dATP, dCTP, dGTP and dTTP.
EDC	Endocrine disrupting contaminant. A natural or man-made substance that has the ability to alter the synthesis, transport, regulation, or clearance of hormones.
E2	Estradiol-17β. A potent estrogenic hormone.

ERS1	Estrogen receptor 1 also named Estrogen receptor alpha. A nuclear receptor which is activated by the binding of estrogens.
ESR2	Estrogen receptor 2 also named Estrogen receptor beta. A nuclear receptor which is activated by the binding of estrogens.
FPT	Female producing temperature. The incubation temperature that results in the development of female gonads in species with environmental sex determination.
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase. An enzyme involved in glycolysis that is commonly used as an internal control gene in mRNA expression studies.
geNORM	Geometric mean normalization. Algorithm used to determine the most stable internal control (housekeeping) genes from a set of tested candidate reference genes in a given sample panel.
GR	Glucocorticoid receptor. A nuclear receptor which is activated by the binding of glucocorticoids.
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1, also called 3β-hydroxysteroid dehydrogenase. A steroidogenic enzyme which converts pregnenolone to progesterone.
HSD17B1	Hydroxysteroid (17-beta) dehydrogenase 1. A steroidogenic enzyme involved in the interconversion of weaker and stronger estrogens and androgens.
LMMs	Linear mixed effects models. A statistical approach that contains experimental factors of both fixed and random-effects types.
MPT	Male producing temperature. The incubation temperature that results in the development of male gonads in species with environmental sex determination.
mRNA	Messenger ribonucleic acid. A type of RNA that is transcribed from DNA in the synthesis of protein.
NF	Normalization factor. A value calculated by geNORM and assigned to each RT-qPCR sample to correct for potential differences in RNA quality or quantity.
NR5A1	Nuclear receptor 5A1, also named steroidogenic factor-1. A transcription factor that regulates genes involved in steroid biosynthesis.

P4	Progesterone. A steroid hormone synthesized from pregnenolone, which plays a critical role in pregnancy.
P5	Pregnenolone. A steroid hormone involved in the steroidogenesis of progesterone, mineralocorticoids, glucocorticoids, androgens, and estrogens.
POLR2E	RNA polymerase II polypeptide E. An enzyme that catalyzes the transcription of DNA in order to synthesize precursors of RNAs.
PR	Progesterone receptor. A nuclear receptor which is activated by the binding of progestins.
PCR	Polymerase chain reaction. A molecular technique used to amplify a section of DNA or cDNA.
RPS13	Ribosomal protein S13. One of the many proteins belonging to the small subunit of the ribosome that is commonly used as an internal control gene in mRNA expression studies.
RNA	Ribonucleic acid. A nucleic acid that is present in all living cells.
RN18S1	18S ribosomal. The small ribosomal unit of RNA that is commonly used as an internal control gene in mRNA expression studies.
RPL8	Ribosomal protein L8. A ribosomal protein that is a component of the 60S subunit of RNA that is commonly used as an internal control gene in mRNA expression studies.
RT-qPCR	Real-time quantitative polymerase chain reaction. A molecular technique for the quantification of an amplified PCR product based on incorporation of a fluorescent reporter dye. The fluorescent signal increases in proportion to the amount of PCR product produced and is monitored at each cycle, such that the time point at which the first significant increase in the amount of PCR product correlates with the initial amount of target template.
StAR	Steroidogenic acute regulatory protein. A transport protein that regulates cholesterol transfer from the outer to inner mitochondria membrane.
T	Testosterone. An androgen steroid hormone.
TSP	Thermo-sensitive period. The period of embryonic development when the developing gonad is sensitive to the effects of incubation temperature.

TSD	Temperature sex determination. A type of environmental sex determination where incubation temperature during development determines the sex of the embryo.
11β-HSD	11β-hydroxysteroid dehydrogenase. Two enzymes (type 1 and 2) associated with the interconversion of cortisol and cortisone.

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

THE EVOLUTION OF ENDOCRINE EXTRAEMBRYONIC MEMBRANES; A COMPARATIVE STUDY OF STEROIDOGENESIS AND STEROID SIGNALING IN THE CHORIOALLANTOIC MEMBRANE OF OVIPAROUS AMNIOTES

By

Lori Cruze Albergotti

December 2011

Chair: Louis J. Guillette, Jr.
Major: Zoology

During development, all amniotes (mammals, reptiles, and birds) form extraembryonic membranes, which regulate gas and water exchange, remove metabolic wastes, provide shock absorption, and transfer maternally derived nutrients. In viviparous (live-bearing) amniotes, both extraembryonic membranes and maternal uterine tissues contribute to the placenta, an endocrine organ that synthesizes, transports, and metabolizes hormones essential for development. Historically, endocrine properties of the placenta have been viewed as an innovation of placental amniotes. However, an endocrine role of extraembryonic membranes has not been investigated in oviparous (egg-laying) amniotes despite similarities in their basic structure, function, and shared evolutionary ancestry.

To begin addressing this question, I examined steroidogenesis and steroid hormone signaling capability in the chorioallantoic membrane (CAM) of the domestic chicken (*Gallus gallus*), the American alligator (*Alligator mississippiensis*) and the Florida red-belly slider turtle (*Pseudemys nelsoni*), representing three lineages of the amniote phylogeny that reproduce strictly by oviparity. To investigate steroidogenesis, I

examined mRNA expression of key steroidogenic enzymes involved in the biosynthesis of steroid hormones by real-time quantitative PCR. Further, I examined the CAMs capability to synthesize progesterone *in vitro* in the presence of a steroid precursor by explant culture and radioimmunoassay. To investigate steroid hormone signaling, I quantified mRNA expression of steroid receptors and confirmed protein expression of two steroid receptors; the progesterone and estrogen receptor by immunohistochemistry.

Collectively, the data presented here indicates that the oviparous CAM is steroidogenic and has steroid hormone signaling capability. These findings represent a paradigm shift in evolutionary reproductive biology by suggesting that endocrine activity of extraembryonic membranes is not a novel characteristic of placental amniotes. Rather, we hypothesize that endocrine activity of extraembryonic membranes is an evolutionarily conserved characteristic of amniotes. If steroidogenesis and steroid signaling in extraembryonic membranes is conserved, this would then suggest that the endocrine role of the amniote placenta likely evolved initially in an oviparous ancestor and offers a new hypothesis for the evolution of the placenta as an endocrine organ.

Despite numerous studies demonstrating the presence of endocrine disrupting contaminants in the reptilian and avian CAM, extraembryonic membranes have not been established as targets of endocrine disruption. In an attempt to better understand the regulation of steroid activity in and the impact of environmental contaminants on these tissues; mRNA expression of steroid receptors and steroidogenic enzymes in the alligator CAM was examined following estrogenic exposure. I observed a change in steroid receptor mRNA expression following exposure to the naturally occurring

estrogen, 17β-estradiol. This suggests that xenoestrogens could interfere with embryonic development through the steroidogenic pathway of the CAM itself; however, more work is needed to better understand the biological impact of environmental contaminants on these tissues.

CHAPTER 1
INTRODUCTION[1]

Since ancient Greece, philosopher-biologists have described similarities and differences in the structures, which support the developing embryo, between oviparous (egg-laying) and viviparous (live bearing) animals [1, 2]. Aristotle, in his treatise, *De generatione animalium* (On the generation of animals), proposed that fetal nutrition in viviparous mammals was accomplished by a connection between the fetal and maternal circulatory systems via the "umbilicus". In describing the chick, he ascertained that the circulatory system of the embryo was also connected via umbilical cords to two membranes; one that surrounded the yolk, and one that lined the inner surface of the eggshell. He suggested that the first membrane delivered nutrients from the yolk for embryonic development, and in comparing viviparous and oviparous animals suggested that "at first this embryo, whether in an egg or in the mother's uterus, lives the life of a plant, for it receives its first growth and nourishment by being attached to something else." In describing the second membrane, which he claimed resembled a "chorion" (Latin for membrane), suggested that "the relation of the chick to the outermost membrane, the sanguineous one, is like that of the mammalian embryo to the uterus" [2].

Subsequently, generations of researchers have dedicated their careers to the study of extraembryonic membranes and the placenta. While studies focused primarily on the extraembryonic membranes of mammals and the chick until the 19th century, we now have an increasing number of descriptions of piscine (reviewed in [3]) and

[1] A significant part of this chapter is published, see Albergotti L, C., Guillette LJ, Jr. Viviparity in reptiles: evolution and endocrine physiology. In: Norris DO, Lopez KH (eds.), Hormones and reproduction in vertebrates, vol. 3: Elsevier; 2011: 247-275.

squamate (lizard and snakes) placentae and extraembryonic membranes (reviewed in [4]. A web of science search reveals that nearly 18,000 scientific papers have been published on the placenta or extraembryonic membranes from 1900 to present and that does not take into account the early works of the pioneers in this field. Yet, even with the staggering amount of research conducted to date, there still remain many unanswered questions about the physiology and evolution of extraembryonic membranes and the placenta [1].

My primary interest concerns the physiology, and more specifically the endocrinology, of extraembryonic membranes and the evolution of endocrine signals in these tissues. Prior to the early 1900s, the placenta was considered to function as an organ of protection, nutrient transfer, gas exchange and waste removal. An endocrine role for the placenta was first proposed by Joseph Halban in 1905 [5] and when Egon Diczfalusy (1955) went on to suggest that the metabolism (and possibly production) of hormones might be a joint effort of the fetus and placenta acting as a “fetal-placental unit” his idea was met with great skepticism [6]. The amazing foresight shown by these early researchers has led not only to a greater understanding of the endocrinology of mammalian pregnancy, but also has led researchers to ask comparative questions about the endocrinology of gestation in nonmammalian viviparous vertebrates.

The Evolution of Viviparity

It is currently estimated that viviparity has evolved independently over 100 times within squamate reptiles [7]. This transition in reproductive mode from oviparity to viviparity has occurred more frequently in squamates [8] than in any other vertebrate lineage including fishes [9, 10], amphibians [11, 12], and mammals [13]. This high frequency of occurrence in squamates coupled with a complete absence of viviparity in

other reptiles [7] has made the study of squamate viviparity intriguing for more than a century.

In order for reptilian viviparity to evolve, viviparous species must overcome three major morphological and/or physiological modifications. First, egg retention time must be increased so that the embryo completes development *in utero*. Second, eggshell thickness must be decreased to reduce the physical barrier between embryonic and maternal environments, so that, at the least, gas and water can effectively be exchanged. Third, a placenta must develop to facilitate maternal and embryonic exchange [14]. Due to the diversity of viviparous squamates and the number of proposed independent origins, these processes could have been achieved through different mechanisms, but then again limited by physiological and morphological constraints. Here, I provide a brief overview of some of the work that has shaped our understanding of these modifications associated with the evolution of viviparity with a focus on the evolution of the placenta. In addition, I will present a new hypothesis for the evolution of endocrine extraembryonic membranes, including the placenta, of amniotes (mammals, reptiles including birds).

Egg Retention and the Lengthening of Gestation

By definition, viviparity requires that the time of egg retention is increased so that the embryo completes development *in utero.* In order to understand the lengthening of gestation in squamates, we must understand the physiology of pregnancy and thus, first turn our attention to the corpus luteum and its possible role in egg retention.

In all vertebrates, the corpus luteum (CL) is a transitory endocrine organ formed from the post-ovulatory follicle. Following ovulation, the remnants of the ovarian follicle's granulosa and thecal cell layers undergo luteinization, which involves changes in

vascularization, hypertrophy, and the accumulation of lipids [15]. The CL functions in the secretion of hormones for some predetermined time period before degenerating (luteolysis). The natural lifespan of the CL varies in vertebrates, but is short-lived in most mammalian species unless implantation occurs. If implantation is successful, the life of the CL is “rescued”, i.e., extended by embryonic factors associated with the maternal recognition of pregnancy [15, 16]. In placental mammals, the maternal recognition of pregnancy is defined as the prevention of luteolysis by biochemical signals of embryonic origin [17], which vary in mammals. Depending on the species, maternal recognition of pregnancy can be provided by a number of embryonic factors, such as chorionic gonadotropins, interferons, steroids, and peptide hormones [17].

The endocrine properties of the CL are most often associated with the secretion of progesterone (P4) in non-mammalian vertebrates [18-21], but the CL has also been shown to secrete other steroid and peptide hormones in eutherian mammals and viviparous squamates [22-24]. In eutherians, P4 is associated with uterine quiescence and as such P4 secretion by the CL plays an important role in the maintenance of pregnancy until synthesis of this steroid hormone is assumed by the placenta in many species [16, 25-27]. The CL is a major source of P4 in reptiles; therefore, it has been suggested that the CL in squamates could provide some hormonal control of gravidity via the secretion of P4 [14, 20, 28].

In the reptiles that reproduce strictly by oviparity, the tuatara, crocodilians, and turtles, the life of the CL is approximately the length of time it takes to shell the eggs (a few days to a few weeks) and it deteriorates shortly before or after oviposition [29, 30] whereas the CL of squamates is maintained for varying periods of gravidity that can last

for weeks or months [31-34]. In oviparous squamates, the presence of an active CL is strongly correlated with egg retention and the timing of oviposition coincides with the loss of luteal activity [14, 35]. However, viviparous squamates show a great deal of variation in the life of corpora lutea with luteolysis occurring well before parturition in some species, which precludes any gross generalizations concerning the role of the CL *alone* in the lengthening of egg retention in viviparous species (as reviewed in [36]).

Maintenance of the CL as well as its correlation with circulating P4 concentrations is also variable in viviparous squamates. Many studies have determined the CL to be the primary source of P4 in viviparous squamates and have reported a strong positive correlation between the persistence of the CL during gestation and circulating P4 concentrations in viviparous lizards [33, 37, 38] and snakes [39, 40]. However, in *Sceloporus jarrovi* [41] and *Chalcides chalcides* [32], plasma P4 concentrations remain elevated late in gestation following luteolysis. In *S. jarrovi*, Guillette et al. (1981) found luteolysis to occur in conjunction with the development of the chorioallantoic placenta. This observation led to the hypothesis that another source of P4 –the placenta -- could be important in the maintenance of gestation [41]. Such a hypothesis mirrors the pattern found in many eutherian mammals in which P4 synthesis by the placenta replaces that of the CL during gestation [16].

The mechanisms controlling the lengthening of egg retention in squamates are still poorly understood. In some species, correlational evidence presents a strong case for the role of the CL in the maintenance of gestation; however, in other species we can find no such correlation. Likewise, circulating concentrations of P4 during gestation are quite variable across viviparous species. In addition, essentially nothing is known about

the maternal recognition of pregnancy in viviparous reptiles, but a few studies suggest that this phenomenon could occur in viviparous lizards [42, 43]. If maternal recognition of pregnancy occurs in reptiles, chemical signals of embryonic origin could function to prevent CL deterioration, modify the uterine environment, and increase the length of gestation. These embryonic chemical signals could be compounds similar to those found in mammals suggesting that the early embryonic expression of cytokines, prostaglandins, estrogens and other steroids as well as peptide hormones should be investigated in oviparous and viviparous squamates [43].

The Reduction of the Eggshell

The reduction of the thickness and structure of the eggshell is another requisite change for the evolution of viviparity in squamates [14, 44]. Oviparous squamates generally lay flexible eggs composed of two layers; a thick inner shell membrane composed of proteinaceous fibers and an extremely thin external crust of calcium carbonate [45, 46]. Some viviparous species also have a shell membrane present for the duration of embryonic development, but an external calcified layer is either absent or greatly reduced [44, 47, 48]. All viviparous species examined demonstrate either a significant reduction in eggshell thickness or loss of the eggshell altogether [49]. However, the debate continues over the timing of eggshell reduction in terms of the evolutionary transition to viviparity. One hypothesis proposes that eggshell reduction was a refinement of viviparity occurring after complete egg retention had evolved whereas the other and more generally accepted hypothesis proposes that a reduction in the eggshell evolved concurrently with increasing periods of egg retention and thus is a requirement of viviparity [14, 47, 50]. The later hypothesis has been supported by

experiments utilizing the lizard, *Lacerta vivipara*, which demonstrates both parity modes in reproductively isolated populations.

Heulin and colleagues successfully crossed individuals in the laboratory from oviparous and viviparous populations of *L. vivpara,* generating hybrid F1 females that laid eggs with incompletely calcified eggshells and an eggshell thickness (21 μM) intermediate between the completely calcified eggshells of oviparous parental population (40 μM) and the eggshell membrane (9 μM) of the viviparous parental population [51, 52]. Further hybridization experiments of *L. vivipara* performed by Arrayago et al. (1996) resulted in oviposition by hybrid F1 females at an embryonic stage of development also intermediate between the oviparous and viviparous populations. This artificial experiment directly connects increased time of egg retention with decreased thickness of the eggshell, although it does not show a causal relationship.

Two nonexclusive hypotheses have been put forth to explain the adaptive significance of evolving reduced eggshell thickness concurrently with prolonged periods of *in utero* egg retention. The first proposes that decreasing the thickness of the eggshell would facilitate gas exchange between maternal and embryonic environments. This would be particularly important in later stages of development as oxygen requirements of the embryos increase drastically in the exponential growth phase of development [50]. However, this hypothesis was not supported when tested in oviparous species from the genus *Sceloporus* [53]. Oviparous *Sceloporus* species exhibit differences in the amount of embryonic development that can be supported *in utero* past the “normal” time of oviposition. Some species, such as *Sceloporus scalaris,*

support advanced embryonic development during extended egg retention experiments, while other oviparous *Sceloporus* species do not. However, in contrast to the above proposed hypothesis, these differences in the possible amount of extended *in utero* development were not related to thickness of the eggshell [53]. A second hypothesis suggests that reducing eggshell thickness would reduce diffusion distance between embryonic and maternal tissues; thereby facilitating the movement of chemical signals from the embryo to the mother that could play an important role in the maternal recognition of pregnancy [14, 49]. In addition, reducing the eggshell thickness would also reduce the diffusion distance for nutrient transfer from mother to the developing offspring.

A common finding when oviparous and viviparous squamate species are compared is that the uterine shell glands are reduced in some capacity and that reduction is correlated with a reduction in eggshell thickness. The mechanism by which this occurs is still poorly understood but likely involves altered estrogen signaling. Uterine shell glands are prominent in all oviparous squamates studied to date, but are absent or greatly reduced in the majority of viviparous species. In the viviparous species with obvious uterine shell glands, a general reduction in size of the shell gland has been reported as in *L. vivipara* [44] as well as a reduction in the thickness of the endometrial stroma as in *Pseudemoia. spenceri* [54]. Shell glands undergo seasonal changes with reproductive cyclicity that match changes in the prominent sex steroid hormones, E2 and P4; therefore future studies are needed to understand the mechanisms associated with shell gland formation and recruitment. Estrogens appear to be central to shell gland recruitment, proliferation, and synthesis of secretory material and ERs serve as key

modulators in this process. Further, the regulation of the expression of other important factors, such as membrane calcium pumps, needs to be examined. While the mechanisms by which uterine shell glands and the eggshell are reduced remains unresolved, there is evidence that reducing the eggshell evolved concurrently with the evolution of extended egg retention and as such should be considered a requirement for the evolution of viviparity in squamates.

The Emergence of the Placenta

Placentation is another key characteristic of viviparity in squamates. In all viviparous squamates examined, placentae are present and thus appear to be required for embryonic survival *in utero* [14]. Here, we define a placenta as a composite structure of maternal and embryonic origin. The embryonic contribution to the placenta consists of one or more of the four extraembryonic membranes (yolk sac, chorion, allantois, and amnion). These membranes function in the transfer of maternally derived nutrients, regulation of gas and water exchange, removal of metabolic wastes, and protection of the developing embryo [55, 56]. These basic requirements are crucial for embryonic survival and development regardless of whether the embryo is encased within a calcified egg in an external nest (oviparous) or maintained within the maternal uterus (viviparous) (Figure 1-1).

Gas and water exchange with the environment is achieved by two vascularized extraembryonic membranes, the chorioallantois and the yolk sac membrane. The chorioallantoic membrane (CAM) is formed by the apposition of the non-vascularized chorion and highly vascularized allantois and serves as the major respiratory organ of the developing embryo. The yolk sac membrane transfers nutrients from the yolk, functions in water exchange and has been proposed to act as a secondary gas

exchange site supplementing the chorioallantois [1, 14, 57]. To facilitate exchange with the environment, these membranes line the inner surface of the eggshell with the surface area of the CAM increasing dramatically during development to meet the gas demands of the embryo. In viviparous species, the loss of the calcified crust and reduction in the protein layer of the eggshell results in apposition of the eggshell or extraembryonic membranes to the vascularized uterus allowing exchange between the embryo and the maternal environment.

Viviparous squamates have two primary types of placentation present during gestation, a chorioallantoic placenta and some form of yolk sac placenta [58]. Both types of placentas are epitheliochorial in squamates reptiles meaning that the extraembryonic membranes are in contact with the uterus through apposition, but the embryonic tissue does not erode and invade the uterine lining [59]. The yolk sac placenta is typically formed at the abembryonic pole during early stages of embryonic development and broadly describes the apposition of omphalopleure (yolk sac) to the uterine epithelium. The chorioallantoic placenta (allantoplacenta) is present later in development at the embryonic pole and is formed by apposition of the CAM to the uterus. Both yolk sac and chorioallantoic placentas demonstrate a wide range of morphological diversity and exhibit varying degrees of complexity across squamates [reviewed in 4].

Four major types of chorioallantoic placentas have been described in squamates and are differentiated by levels of morphological complexity. Weekes [60] described the first three of these types and a fourth has been proposed by Blackburn and Vitt [4] The simplest and most common type of chorioallantoic placenta, Weekes type I, involves

indirect apposition of the CAM to the uterus without any anatomical specializations in either structure. A reduced shell membrane encasing the embryo is present and it is this structure that lies in contact with the uterine epithelium. In types II and III, we see the emergence of the placentome, a specialized region that forms at the embryonic pole and brings the chorionic epithelium and uterine epithelium in direct apposition. Here, the chorioallantoic epithelium interdigitates with the uterine mucosa, and this region has been proposed as a site of histotrophic transfer due to the appearance of absorptive chorionic cells and a secretory uterine epithelium [61]. The type IV chorioallantoic placenta is the most complex and has been described in the South American lizard genus *Mabuya* [62]. This type of chorioallantoic placenta undergoes extensive transformation and demonstrates invagination of the CAM by uterine endometrium forming interlocking projections. The placentome and specialized chorionic cells called areolae have been suggested to function in nutrient transfer [62].

Oviparous species and viviparous species with a simple placenta (type I) are predominately lecithotrophic, meaning that nourishment for embryonic development is primarily supplied by the yolk [63]. Therefore, in the transition to viviparity as the period of egg retention was increased and eggshell thickness decreased, the major requirement of a placental structure was to aid in gas exchange, which appears to be the primary role of the type I placenta. Species forming a more complex chorioallantoic placenta exhibit differentiation into specialized regions, the placentome and paraplacentome. The paraplacentome has been described as possibly functioning as a specialized gas exchange organ in *Pseudemoia entrecasteauxii* [64] and as another site of histotrophic transfer in *Mabuya mabouya* [65]. While the vast majority of viviparous

squamates are lecithotrophic, it is common for these species to exhibit some degree of placental transport of organic and inorganic nutrients as well [66-69].

In contrast, the condition of matrotrophy is quite rare in squamates. Yet, a few species exhibit a high degree of matrotrophy in which the majority of the nutrients required for embryonic development are provided by the mother via a placenta. These species have microlecithal eggs with little or no yolk present in the oocyte. The New World species of the genus *Mabuya* demonstrate the most extreme form of matrotrophy, which surpasses that found in any other squamate reptile and is similar to that of eutherian mammals [65]. The specialized type IV chorioallantoic placenta of *Mabuya heathi* provides approximately 99% of nutrients for embryonic development [70].

An Endocrine Placenta

In eutherian mammals, the placenta is an endocrine organ that synthesizes, metabolizes, and transports a suite of steroid and peptide hormones crucial for embryonic survival and development [71]. P4 is a key placental hormone that plays a role in the maintenance of pregnancy [72], timing of birth [73], and promotes growth of the embryo [74] and of the placenta itself [74-76]. The first indirect evidence of placental P4 synthesis in squamates came from observations in *S. jarrovi*. In this lizard species, maternal plasma P4 concentrations remain elevated after luteolysis and because luteolysis occurs in conjunction with the development of the chorioallantoic placenta, this led to the hypothesis that another source of P4 - the placenta - could be important in the maintenance of gestation [41].

The three-toed skink, *Chalcides chalcides* also demonstrates elevated plasma P4 following deterioration of the CL and formation of the placenta. The chorioallantoic

placenta of this species has been shown to be capable of steroidogenesis [32]. Guarino et al. (1998) investigated the expression of the steroidogenic enzyme hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1), which converts pregnenolone (P5) to P4, in the CL and chorioallantoic placenta. They found high HSD3B1 activity in the CL during early gestation, but not at late pregnancy, whereas the placenta exhibited intense HSD3B1 only at late pregnancy. *In-vitro* synthesis of P4 by the CL was high during early, but not late pregnancy, but P4 synthesis was highest at late pregnancy in the placenta. This work supports the hypothesis of an endocrine chorioallantoic placenta in *C. chalcides* and models a similar pattern found in many eutherian mammals where the placenta assumes the primary role of P4 synthesis following deterioration of the CL [32].

In-vitro production of P4 by the chorioallantoic placenta also has been demonstrated in the southern snow skink (*Niveoscincus microlepidotus*). Girling and Jones (2003) reported that the chorioallantoic placenta of this species was capable of synthesizing P4 when P5 precursor was added to the culture media; however, unlike *C. chalcides* this study did not find significant levels of P4 produced by the placenta in the absence of added P5 [21].

The relatively simple, type I chorioallantoic placenta of *S. jarrovi* can perform *in vitro* metabolism of steroid hormones and synthesis of P4 in the presence of P5 [77]. *In-vitro* experiments revealed that placental tissue rapidly clears P4 and corticosterone (CORT) from culture media, indicating the ability of the placenta to convert P4 and even corticosteroids to other metabolites [77]. Painter and Moore (2005) suggested that steroid hormone metabolism by the squamate chorioallantoic placenta could play an

important role in the regulation of maternal and embryonic hormone concentrations by preventing steroid hormones from freely diffusing between the developing embryo and mother.

The extraembryonic membranes of oviparous species and placentae of viviparous species perform similar functions to meet the gas, water, and nutrient demands of the embryo (Figure 1-1). This indicates that very little specialization of extraembryonic membranes is required in a transition to viviparity and explains why the majority of viviparous species exhibit a relatively simple chorioallantoic placenta that appears to predominately function in gas exchange. Yet, evidence indicates that the chorioallantoic placenta has additional roles in endocrine processes in squamates. Are these specializations unique to the chorioallantoic placentae of viviparous mammals and squamates or could the CAM of oviparous species also perform similar functions?

A New Hypothesis for the Evolution of Endocrine Extraembryonic Membranes and the Placenta: Implications for the Evolution of Viviparity

We have suggested previously [36], that endocrine properties of chorioallantoic placenta are not novel features of viviparous mammals and squamates. Rather, a hypothesis was presented stating that the oviparous CAM has endocrine properties, which gave rise to an endocrine placenta. The basis for our hypothesis comes from studies investigating mammalian [71, 78, 79] and lizard [21] placentae, which revealed that both extraembryonic membranes and maternal tissues contribute to hormone synthesis and metabolism. Given that extraembryonic membranes share numerous similarities in their basic structure and function that are conserved across amniotes [55], I hypothesize that the oviparous CAM is an endocrine organ that has the capability to synthesize and respond to steroid hormone signaling.

If the CAM of oviparous amniotes is capable of producing and responding to embryonic signaling factors, such as steroid hormones, these signaling factors would have functions other than the maternal recognition of pregnancy in oviparous species and could likely be involved in tissue proliferation, vascularization, and embryonic growth. However, these factors would be useful embryonic signals of pregnancy as viviparity evolves and endocrine properties of oviparous species could become co-opted in a novel environment such as the uterus; thus, facilitating the use of embryonic signaling factors in the maternal recognition of pregnancy in viviparous squamates. That is, as squamates retained the egg *in utero* for longer time periods and eggshell thickness was reduced, signaling factors synthesized by the extraembryonic membranes would be free to communicate with the maternal uterus and could play a role in maintaining the life of the corpus luteum and further lengthen gestation until "hatching" occurred *in utero*.

Specific Hypotheses

My aim is to determine if steroidogenic activity of extraembryonic membranes is a unique characteristic of placental amniotes, or if these membranes also have an endocrine role in oviparous non-mammalian species. To investigate this question, I will examine steroidogenesis in the CAM of taxa representing the major phylogenetic groups of non-mammalian amniotes, which reproduce strictly by oviparity. I specifically test the following hypotheses: (1) the molecular mechanisms required to perform steroid hormone synthesis are evident in the oviparous CAM (Figure 1-2); (2) the CAM has the capability to synthesize steroid hormones; (3) the molecular mechanisms required to respond to steroid hormone signals are present in the CAM; (4) the CAM has the capability to modulate steroid hormone signals; (5) mRNA expression in the CAM is

regulated and levels of steroidogenic enzymes and/or steroid receptors can be altered by exposure to endocrine disrupting contaminants (EDCs) during embryonic development.

Significance of Work

The presence of steroidogenic activity in the extraembryonic membranes of oviparous amniotes would imply a more ancient origin of endocrine activity for these membranes than is currently believed. This serves as the first major work to investigate steroidogenesis and steroid signaling in the CAM of oviparous amniotes. By focusing on three of the major phylogenetic groups of non-mammalian amniotes that reproduce strictly by oviparity (birds, crocodilians, and turtles), I attempt to uncover a potentially conserved trait within amniotes. In addition, I attempt to determine whether steroidogenic activity in the CAM is regulated. It is vital to the understanding of this system to reveal this distinction because if gene expression in the CAM is regulated, it is possible for gene expression to be altered. EDCs have been identified in the CAMs of crocodilians, turtles, and birds [80-82]. Aside from identifying their presence in the CAM, it is not currently known whether EDCs could disrupt normal CAM function. However, if endocrine activity of the CAM is a conserved trait of amniotes and if steroidogenesis is regulated in this tissue, EDCs could potentially interfere with steroidogenesis and steroid signaling in the CAM during development.

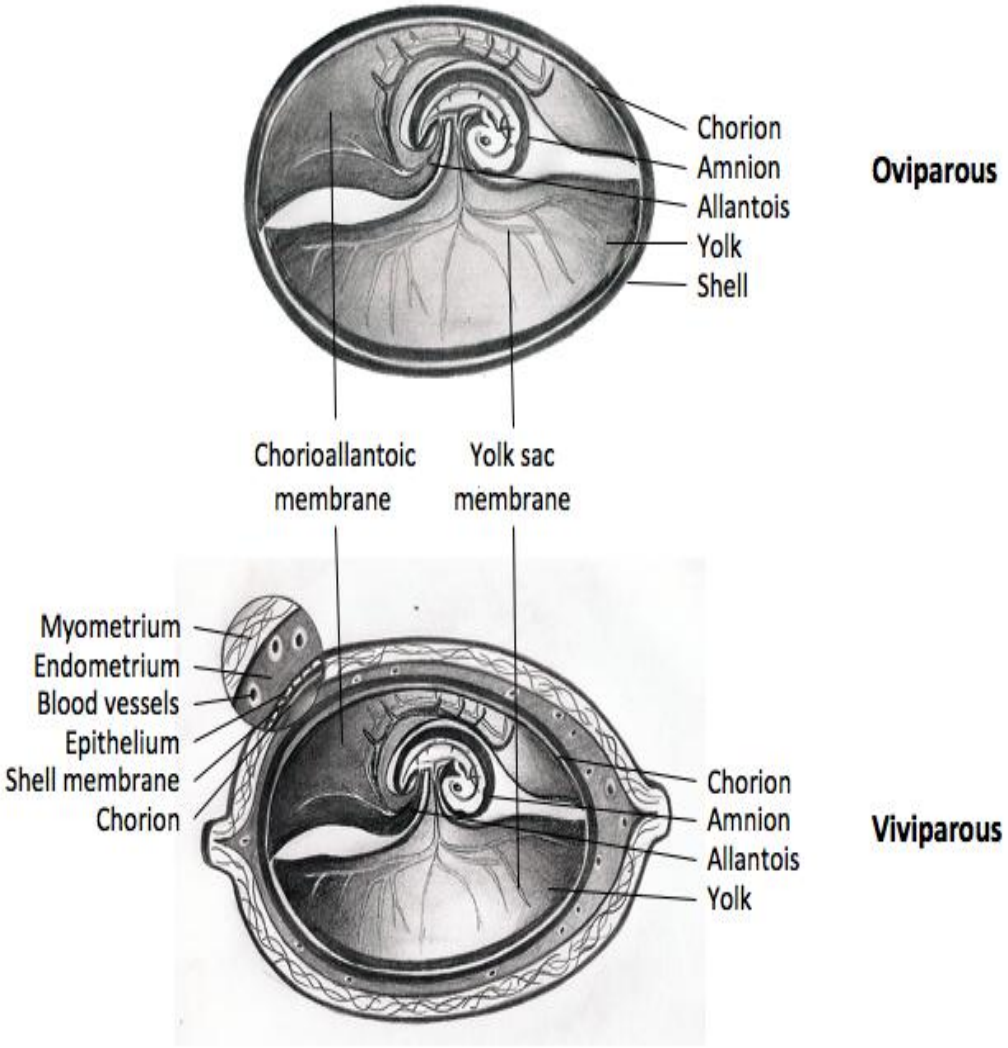
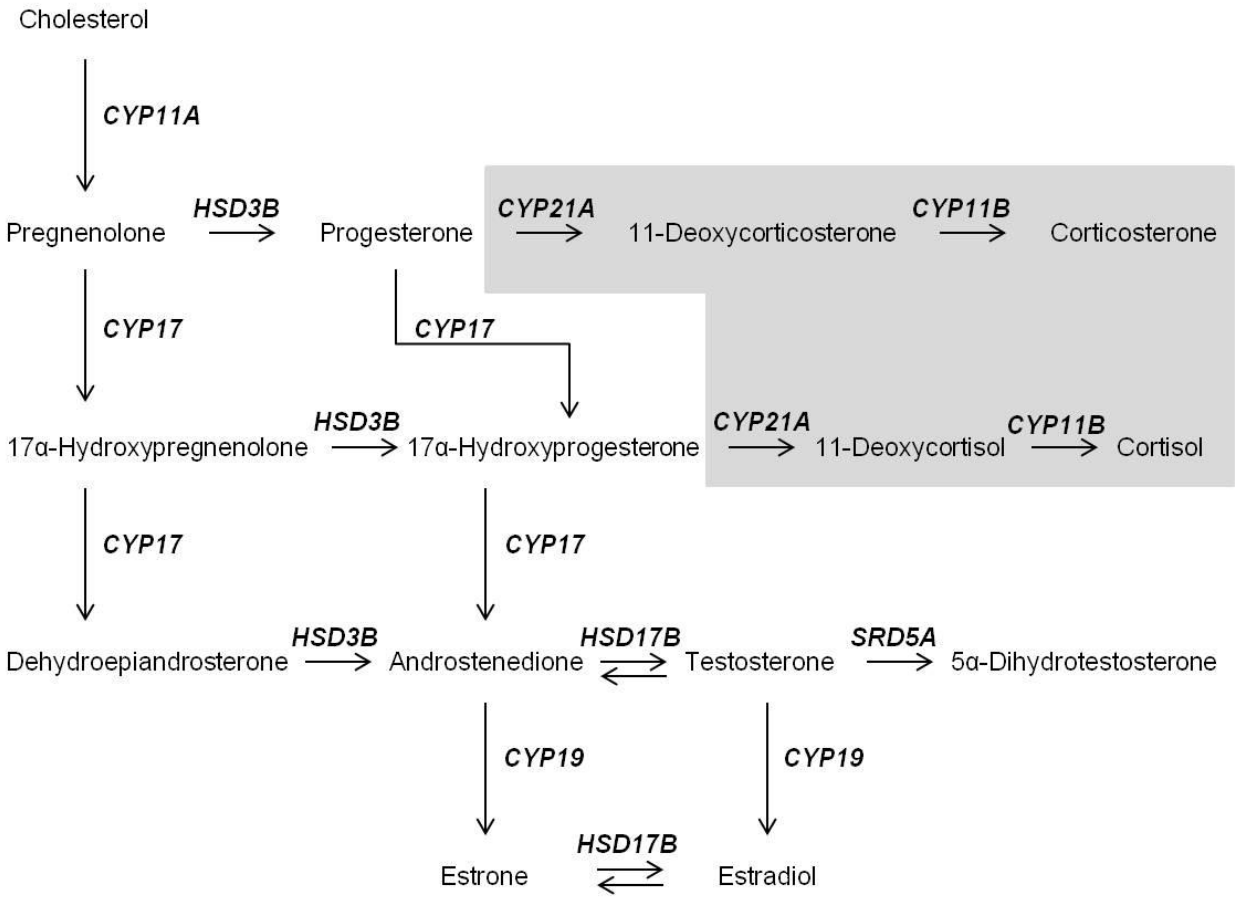


Figure 1-1. The extraembryonic membranes of oviparous and viviparous squamates. Oviparous and viviparous amniotes share the same extraembryonic membranes, the amnion, chorion, allantois, and yolk sac. These structures perform similar functions regardless of reproductive mode indicating that very little specialization of extraembryonic membranes is required in an initial transition to viviparity. Figure redrawn from Guillette LJ, Jr. The evolution of viviparity in lizards. Bioscience 1993; 43:742-751. Artwork by Patpilai Kasinpila.



CYP11A	Cytochrome P450 11A1, also called side chain cleaving enzyme
CYP11B	Cytochrome P450, family 11, subfamily B, also called 11β-hydroxylase.
CYP17	Cytochrome P450, family 17, also called 17α-hydroxylase or 17,20 lyase.
CYP21B	Cytochrome P450, family 21, subfamily B, also called 21-hydroxylase.
CYP19	Cytochrome P450, family 19, subfamily A, polypeptide 1, also called aromatase
HSD3B	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1, also called 3β-hydroxysteroid dehydrogenase.
HSD17B	Hydroxysteroid (17-beta) dehydrogenase 1. also called 17β-hydroxysteroid dehydrogenase.
SRD5A	Steroid-5-alpha-reductase, alpha polypeptide, also called 5α-reductase.

Figure 1-2. General overview of the steroid hormone biosynthetic pathway. Cholesterol is the substrate for *de novo* steroid hormone synthesis and steroidogenesis proceeds by the conversion of one hormone to another by the action of specific enzymes (italicized general class abbreviations shown above arrows, see legend for full gene names). The grey shaded region reflects the stress hormone pathway; whereas un-shaded regions depict synthesis of sex steroid hormones.

CHAPTER 2
STEROIDOGENESIS AND STEROID SIGNALING IN THE CHORIOALLANTOIC MEMBRANE OF THE CHICKEN (*GALLUS GALLUS*)[2]

A key defining characteristic of amniotes (mammals, reptiles, and birds) is the formation of four extraembryonic membranes during embryonic development; the amnion, chorion, allantois, and yolk sac [55]. Fusion of the chorion and allantois forms either the chorioallantoic placenta in viviparous (live-bearing) species, or the chorioallantoic membrane (CAM) in oviparous (egg-laying) species [83]. Both the chorioallantoic placenta and CAM perform functions crucial for embryonic survival and development [55, 83]. Yet, only the placenta, which is a composite structure composed of extraembryonic membranes and maternal decidua, is classified as an endocrine organ [71, 83].

The mammalian chorioallantoic placenta synthesizes, transports, and metabolizes a suite of steroid and peptide hormones [71, 83, 84]. Of these, placental progesterone (P4), plays a key role in the maintenance of pregnancy [72], timing of birth [73], and promotes growth of the embryo [74] and placenta [75, 76]. Historically, endocrine properties of the placenta have been viewed as an innovation of eutherian mammals [83]. However, evidence of an endocrine placenta in three species of viviparous lizards [21, 32, 77] has recently called this traditional eutheriancentric view into question.

Examination of mammalian [71, 78, 79] and lizard [21] placentas has revealed that both extraembryonic membranes and maternal tissues contribute to hormone synthesis and metabolism. Therefore, I asked whether the extraembryonic membranes

[2] Published previously, see Albergotti LC, H.J. Hamlin, M.W. McCoy, L.J. Guillette, Jr. Endocrine Activity of Extraembryonic Membranes Extends beyond Placental Amniotes. PLoS ONE 2009; 4(5):e5452.

of oviparous amniotes could also play a role in the production of hormones during embryonic development. Although some differences do exist in the formation of the chorionic ectoderm between placental and oviparous amniotes [85], such an investigation is warranted given that the extraembryonic membranes share numerous similarities in their basic structure and function that are conserved across amniota. The presence of steroidogenic activity in the extraembryonic membranes of an oviparous amniote would imply a more ancient origin of endocrine function for these membranes than is currently believed.

Both the chorioallantoic placenta and CAM are derived from chorion and allantois [83, 85]. Therefore, if the CAM has similar steroidogenic properties as the chorioallantoic placenta, then it should synthesize key placental hormones, such as P4. Here I examined the potential activity of P4 in the CAM of chicken (*Gallus gallus*). To confirm P4 activity I must demonstrate that: (1) the oviparous CAM has the molecular mechanisms in place to perform steroidogenesis and synthesis of P4. Indeed, I show mRNA expression patterns of key steroidogenic enzymes involved in P4 biosynthesis. (2) The CAM is able to synthesize P4. I demonstrate that *in vitro* P4 synthesis takes place in the CAM and is not a product of steroids in the yolk or embryo, by isolating the CAM from other tissues and testing for synthesis directly in the presence of a steroid hormone precursor. (3) The CAM is capable of responding to the P4 signal through an appropriate receptor. Again, I demonstrate, via mRNA expression and protein immunolocalization of the progesterone receptor, that the CAM is capable of modulating P4 activity.

Materials and Methods

CAM Collection

Fertilized chicken (*Gallus gallus*) eggs were obtained from Charles River Laboratories (North Franklin, CT) and staged according to Hamburger and Hamilton [86]. CAMs were collected by removing the eggshell directly over the embryo and excising CAM away from embryo and yolk sac membrane. CAMs were washed in 1X phosphate buffered saline (PBS).

RNA Isolation and Reverse Transcription

Dissected CAM was stored in the RNA preservative, RNA*later*® solution (Ambion) at 4°C. Total RNA was isolated from CAM with TRIzol® reagent (Invitrogen Life Technologies), purified with the SV Total RNA Isolation System (Promega), and reverse transcribed with the iScript™ cDNA Synthesis Kit (Bio-Rad). Concentrations and quality of RNA samples were evaluated by measuring optical density with a NanoDrop™ ND-1000 (Thermo Scientific) and by formaldehyde gel electrophoresis. Total RNA was treated with ribonuclease-free deoxyribonuclease I (DNase I; Qiagen) to remove any contamination of genomic DNA. 1 µg of total RNA was reverse transcribed and complementary DNA (cDNA) was diluted 10-fold and stored at -20°C until RT-qPCR analysis.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR analysis was performed on CAM samples from embryonic days 8 (n=10), 10 (n=17), 12 (n=14), 14 (n=10), 16 (n=14), and 18 (n=12). cDNA was analyzed in triplicate by RT-qPCR amplification using an iCycler MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). Each 15-µL DNA amplification reaction contained a 10-fold dilution of 10X Gold Buffer (Applied Biosystems), 3 mM $MgCl_2$, 200 µM dNTPs,

0.04% Tween-20, 0.4% glycerol, 1% DMSO, 500-fold dilution of SYBR Green (Invitrogen), 0.01 μM Fluorescein Calibration Dye (Bio-Rad), 0.2 μM of each primer, 0.67 μL of diluted cDNA, and 0.01 U/μL AmpliTag Gold DNA polymerase (Applied Biosystems). RT-qPCR amplification conditions included an enzyme activation step of 95°C (10 min) followed by 40 cycles (reference genes) or 50 cycles (target genes) of 95°C (15 sec) and a primer specific combined annealing/extension temperature (1 min). The specificity of amplification was confirmed by melt-curve analysis. Triplicate data for each gene were averaged and amplification was determined by the absolute quantification method [87]. In brief, copy numbers were calculated from the cycle threshold value by the linear regression of a standard curve. Standard curves for each target gene were generated from a plasmid containing the amplicon of interest. Controls lacking cDNA template were included on every RT-qPCR plate to determine the specificity of target cDNA. Additionally, to confirm that target cDNA was not contaminated by genomic DNA, RT-qPCR was performed with ACTB and PR primers on the RNA isolated from every sample. To normalize mRNA expression levels, RT-qPCR was performed on all samples with five reference genes: actin, beta (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L8 (RPL8), RNA polymerase II polypeptide E (POLR2E), and ribosomal protein S13 (RPS13). The geometric mean was calculated according to geNORM [88] generating a normalization factor (NF) for each sample to correct for potential differences in RNA quality or quantity. For each target gene, absolute copy number was divided by the NF. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript number in copies/μL ± SEM.

Cloning and Sequencing of Plasmids

RT-qPCR of pooled cDNA was used to generate a PCR product for each primer set. Amplified PCR products were separated on a 2% agarose gel and visualized by ethidium bromide on a Gel Doc EQ with Quantity One 4.6 software (Bio Rad). RT-qPCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega) and purified samples were confirmed by electrophoresis on a 2% agarose gel. PCR products were cloned into a pGEM®-T Vector System (Promega). Plasmid DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega) and sequenced on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) using a BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). The specificity of cloned DNA was confirmed using BLAST against sequences available in Genbank. Clone DNA concentration was quantified by NanoDrop™ ND-1000, converted to copies/µL, and serially diluted in a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 5 µg/mL of tRNA.

RT–qPCR Primers

All Primers were designed to amplify mRNA-specific fragments from chicken coding sequences (National Center for Biotechnology Information) using Primer3 software [89] and were synthesized by Eurofins MWG Operon. Primer pairs were combined and diluted to a final concentration of 10 µM. Primer sets with specific combined annealing/extension temperature used in RT-qPCR are listed in Table 2-1.

Sexing of Embryos

To sex individuals used in RT-qPCR analysis, CAM genomic DNA was extracted from TRIzol® reagent (Zhu, Shirley, DNA extraction from TRIZOL organic phase, http://med.stanford.edu/labs/vanderijn/Protocols.html). DNA concentration was

determined by Nanodrop and diluted to 100 ng/μL. Molecular sexing was performed as described in [90]. For *in vitro* tissue culture, day 18 individuals were sexed by visual inspection of embryonic gonads.

In Vitro Explant Culture

CAMs from 7 eggs were collected on embryonic day 18. CAMs were cut into 12 sections of approximately 0.1g wet weight (mean=0.104g ± 0.0009 SEM) allowing for duplicate sections to undergo identical treatment regimes. CAM sections were incubated at 37°C on an orbital shaker in L-15 culture media (Invitrogen) either with or without cholesterol and cAMP as precursor. Precursor solutions and concentrations are based on King et al. 2004 [91]. For cholesterol, 22(R)-Hydroxycholesterol (Sigma) was dissolved in 95% ethanol (Fisher) to a final concentration of 10μg/mL and combined with 1 mM Dibutyryl cAMP (Sigma). After 2, 4, or 8 hours of incubation, concentration of progesterone in the culture media was quantified by solid phase radioimmunoassay [92]. To determine background and cross-reactivity of the P4 assay, controls consisting of only cholesterol and cAMP were incubated for 8 hours with an average P4 concentration of 0.337pg/mL/g ± 0.423 SEM.

Immunohistochemistry and Microscopy

Dissected CAM was fixed in 4% paraformaldehyde at 4°C overnight. Tissues were washed 3X in 1X PBS and stored in 75% ethanol at 4°C. CAMs were dehydrated, paraffin embedded, and sectioned at 8 microns. Tissue sections were deparaffinized in citrosolv and rehydrated through graded concentrations of ethanol to 0.1 M Tris buffered saline (TBS, pH 7.6). Immunohistochemistry was performed using the Vectastain® Universal Quick Kit, R.T.U. (Vector Laboratories) with the following modifications: for antigen retrieval, slides were autoclaved for 30 min in 10 mM sodium citrate buffer (pH

6.0). Sections were treated with 3% hydrogen peroxide for 20 min, blocked in normal horse serum (NHS) for 1hour, and treated with the Avidin Biotin blocking kit (Vector Laboratories). Between all incubation steps, slides were washed in TBS (5 min), 0.1 M TBS containing 0.2% Tween 20 (5 min), and again in TBS (5 min). CAM sections from day 16 (n=5) and day 18 (n=5) were incubated with a 1:50 dilution of mouse monoclonal anti- progesterone receptor antibody (Ab-8), Thermo Scientific. PR Ab-8 recognizes both PR isoforms in chicken oviduct [93] and ovary [94]. Sections were treated with 3, 39-diaminobenzidine for 5 min (Vector Laboratories) and washed in running tap water for 5 min. A control section receiving normal horse serum in place of primary antibody was included on every slide. Slides were dehydrated, cleared and mounted with Permount™ mounting media (Fisher Scientific). Sections were imaged using a Leica DMRE microscope under DIC and Leica DFC 300 FX camera with Leica Firecam software.

Statistical Analysis

All statistical analyses were performed in the R statistical programming environment version 2.8.0 [95]. For gene expression analyses, the total numbers of mRNA transcripts for 5 control and 5 target genes from the CAM were determined by RT-qPCR. To quantify relative expression of target genes, we divided each sample by a normalization factor to yield normalized quantities (copies/μL). Normalization factors were estimated as the geometric mean expression of 5 control genes using geNORM [88]. Samples displaying a non-specific melt curve were excluded from the analysis and account for differences in number of samples between genes. To analyze each target gene, we used linear mixed effects models (LMMs) to estimate the parameters for relative mRNA expression over the 6 day experiment. For each analysis, embryonic day

was treated as a fixed effect, and embryonic day, RT-qPCR plate, and sex of individuals were treated as random effects. Model assumptions were evaluated visually via examination of residuals and QQ plots and square-root transformations were performed when necessary to normalize errors (all genes were square root transformed except CYP17A1 which did not require transformation). Outliers were identified from residuals and QQ plots and removed from the study (note that inclusion of outliers did not change patterns of significance, but were excluded from the final analysis because they have a disproportionate influence on mean estimates and caused violations of normality). The assumption of homogeneity of variances was met for all genes except HSD3B1, which is likely due to the substantial changes in mean expression of HSD3B1 as the embryo developed. Thus for HSD3B1 variance was assumed to be a power of the estimated mean for each day and the exponent was estimated from the data as part of the estimation procedure [96].

For *in vitro* tissue culture, I used the same analytical approach described above using a LMM to estimate P4 concentration in culture media. In this analysis treatment (precursor versus control) and time (hour) are fixed effects on P4 concentrations (pg/mL/g of CAM tissue) and day of dissection, egg, replicate and sex of individual were treated as random effects. P4 concentration was square root transformed and two outliers, the largest value for control at 4 hours (206) and the largest value for precursor at 8 hours (213) were excluded from the analysis as outliers.

Results

The Chicken CAM has the Required Molecular Mechanisms to Perform Steroidogenesis and Synthesis of Progesterone

Cholesterol is required for the *de novo* synthesis of steroid hormones with steroidogenesis proceeding by the conversion of one steroid hormone to another by the action of specific enzymes [84]. To determine whether the oviparous CAM has the molecular mechanisms required for P4 synthesis, I examined mRNA expression of key steroidogenic enzymes in the steroid biosynthesis pathway [84, 97] (Figure 2-1). The relative levels and timing of mRNA expression were determined by harvesting CAM tissue, which forms on embryonic day 5, on embryonic days 8, 10, 12, 14, 16, and 18 and performing quantitative real-time RT-PCR (RT-qPCR) of mRNA coding for steroidogenic acute regulatory protein (StAR), cytochrome P450 11A1 (CYP11A1), cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1), and hydroxysteroid (17-beta) dehydrogenase 1 (HSD17B1).

I show the presence of steroidogenic enzyme mRNA in the CAM of an oviparous amniote (Figure 2-2). Overall, there was significant mRNA expression of StAR ($F_{1, 67}$=65.222, p< 0.0001), CYP11A1 ($F_{1, 68}$=58.489, p<0.0001), and CYP17A1 ($F_{1, 66}$=80.004, p<0.0001); however, the level of expression did not change significantly between embryonic day 8 and 18 for several components of the steroidogenic pathway (StAR $F_{5, 67}$=1.144, p=0.346), (CYP11A1 $F_{5, 66}$=1.618, p=0.167), (CYP17A1$F_{5, 66}$=1.787, p=0.128). In contrast, I observed a 464 fold increase in HSD3B1, which converts pregnenolone (P5) to P4, from day 8 to day 18 of development ($F_{5, 68}$=89.282, p<0.0001). Detection of the steroidogenic enzymes required for the transport and

conversion of cholesterol to P4 identifies a molecular mechanism for achieving P4 synthesis in the CAM. Additionally, the significant increase in HSD3B1 indicates that P4 synthesis in the CAM potentially increases through development similar to that of the chorioallantoic placenta through pregnancy [84, 98].

In contrast, HSD17B1 showed a significant decrease in expression, with mRNA expression decreasing to nearly zero by day 18 ($F_{5, 68}$=16.027, p<0.0001), which could be associated with decreasing yolk androgens during development. Early in chick development, Elf and Fivizzani [99] reported high levels of androstenedione (A), testosterone (T), and dihydrotestosterone in the yolk, which decreased as development proceeds. They showed that yolk estradiol-17β remained constant through development until increasing on embryonic day 20, which is beyond the duration of our study. Because HSD17B1 is a key enzyme in the conversion between A and T [100] (Figure 2-1), high levels of yolk A and T earlier in embryonic development could explain why HSD17B1 mRNA expression in the CAM was initially high and then decreased through time. This scenario suggests that the CAM utilizes a maternal source of androgens during development. Taken together, our results indicate that the chick CAM has the molecular mechanisms in place to perform steroidogenesis in general and P4 synthesis in particular.

The Chicken CAM is Capable of *In Vitro* Progesterone Synthesis

Placental P4 synthesis in mammals is generally elevated from mid to late pregnancy [98]; therefore, to investigate P4 synthesis in the CAM, I harvested CAM tissue on embryonic day 18 and performed *in vitro* explant culture. Sections of CAM were incubated in culture media for 2, 4, or 8 hours either with or without cholesterol (plus cAMP) as a precursor. The concentration of P4 in the culture media was then

quantified by radioimmunoassay. If the CAM is steroidogenic, addition of the steroid hormone precursor (cholesterol) to the culture media should stimulate increased P4 production. Indeed, our results showed a significant increase in concentration of P4 in the culture media following the addition of cholesterol precursor ($F_{1, 58}$=46.917, p<0.0001) (Figure 2-3). Additionally, I observed a significant interaction between time of incubation and addition of precursor to the culture media ($F_{2, 58}$=3.709, p= 0.0305). This result confirms that P4 synthesis can be induced in the chick CAM in the presence of a steroid hormone precursor. Further, I found that in the absence of precursor, the CAM produced a detectable level of P4 that did not change significantly during the assay (mean=34 pg/mL/g ±8.2 SEM, $F_{2, 21}$=1.626, p=0.2205) (Figure 2-3) suggesting that the CAM can exhibit endogenous P4 synthesis, but under the *in vitro* conditions used here this synthesis is likely limited by the lack of precursor. In contrast, a decrease in P4 concentration during the assay would have indicated that P4 detected at the first time point was perhaps the product of hormones leaching from this highly vascularized tissue. In sum, our results demonstrate that the chick CAM is capable of *in vitro* P4 synthesis. At present, I am unable to comment on the bioavailability of cholesterol in the CAM under *in vivo* conditions as these data do not currently exist.

The Chicken CAM is Capable of Responding to P4 Signaling Through the Progesterone Receptor

Finally, I examined the capability of the chick CAM to respond to P4 signaling through an appropriate hormone receptor. As in human [101], chick P4 receptor (PR) is predominately expressed in two isoforms, PR-A and PR-B [102]. In chicken, the mRNA sequences of these isoforms are identical with the exception that PR-B has an additional 128 amino acids located at the N-terminus [102]. To identify both PR isoforms

in the CAM, I designed primers that recognized the shared mRNA sequence (PR) and performed RT-qPCR to examine relative expression during development. I show that PR increased significantly through embryonic development ($F_{5, 68}$=15.897, p<0.0001), demonstrating a 758% increase between embryonic day 8 and 18 (Figure 2-4A). I hypothesized that the observed increase in PR expression in the CAM could be due to autoregulation by P4 and/or upregulation by an estrogen.

If CAM PR is under autoregulation, i.e. P4 regulates its own synthesis, one might expect that as HSD3B1 and presumably P4 increases, a corresponding decrease in PR expression would result [103]. However in placental tissues, P4 has been shown to maintain and possibly upregulate PR in rats [76], and to significantly increase the expression of PR in humans [104]. Therefore, it is possible that an increase in HSD3B1 and P4 could result in an increase in PR expression in the CAM. If CAM PR is upregulated by an estrogen, I might expect to see an upregulation of both chick PR [105] and estrogen receptor alpha (ESR1) [106]. Therefore, I examined ESR1 mRNA expression in the CAM and found that ESR1 increased by 307% between embryonic day 8 and 18 ($F_{5, 66}$=14.432, p<0.0001) (Figure 2-4A) suggesting that an estrogen might play a role in PR regulation.

To determine if PR mRNA is translated at the level of the protein I performed immunolocalization of the nuclear PR with an antibody designed to recognize both chicken A and B isoforms [93]. I found PR to be localized to the nucleus predominately in the chorionic epithelium and in the epithelial cells of mesenchymal blood vessels (Figure 2-4B). Positive nuclear staining was also found in the allantoic epithelia and

mesenchyme. In total, mRNA expression and protein localization of PR in the CAM indicates that this tissue can modulate the activity of P4 during embryonic development.

Discussion

Collectively, our study indicates that the chick CAM is steroidogenic and has the capability to both synthesize progesterone and receive progesterone signaling. By demonstrating mRNA expression of steroidogenic enzymes, I show that the chick CAM has the molecular mechanisms in place to perform steroidogenesis and biosynthesis of P4. I demonstrate that the CAM is capable of *in vitro* synthesis of P4 in the presence of a steroid precursor. Additionally, I show that the CAM is capable of modulating P4 activity through the progesterone receptor.

In eutherian mammals, placental P4 plays a key role in the maintenance of pregnancy [72], timing of birth [73], and promotes growth of the embryo [74] and of the placenta itself [74-76]. Further, P4 has been observed to stimulate blood vessel proliferation [107] and maturation [108] in the mouse endometrium. Additionally, P4 has been suggested to play a role in human fetoplacental vascularization by regulating the proliferation of placental vascular smooth muscle cells [109] and through relaxation of placental blood vessels [110]. At present, I can only speculate on the role of P4 in the physiology of the CAM. I hypothesize that P4 in the oviparous CAM might be expected to serve similar roles as in eutherians contributing to the maintenance of embryonic development, timing of hatch, and growth of the embryo and of the CAM. Like the placenta, the CAM is a highly vascularized organ; therefore, I suggest that P4 might be involved in CAM blood vessel proliferation and maintenance.

Our findings represent a paradigm shift in evolutionary reproductive biology by indicating that steroidogenic activity of extraembryonic membranes is not a novel

characteristic of placental amniotes. Further, I hypothesize that endocrine activity of extraembryonic membranes might be an evolutionarily conserved characteristic of amniotes. If steroidogenic activity of extraembryonic membranes is evolutionarily conserved, then the endocrine role of the amniote placenta is likely to have evolved initially in an oviparous ancestor.

Our hypothesis that the extraembryonic membranes of amniotes are steroidogenic suggests an additional unifying characteristic of amniotes and has implications for evolutionary reproductive biology, particularly for the evolution of viviparity. It is currently estimated that within squamates (lizards and snakes) viviparity has independently evolved approximately 105 times [8]. In the transition from oviparity to viviparity in squamates, the period of time that eggs are retained within the uterus is increased and the thickness of the eggshell is decreased [14]. Eggshell reduction facilitates maternal-fetal gas exchange, but has also been proposed to function in the diffusion of chemical signals from the embryo to the mother in order to prolong gestation [14]. Thus, secretion of steroid hormones by the oviparous CAM could be important in establishing maternal recognition of pregnancy during the evolution of viviparity in this group. I suggest that evolutionary tinkering in the timing and spatial expression of steroidogenic genes in the CAM could lead to novel endocrine functions in communication with the maternal uterus; thus, facilitating the endocrine role of the chorioallantoic placenta.

Table 2-1. Chicken PCR primers used for RT-Quantitative real-time PCR

Gene	Direction	Sequence (5'-3')	Annealing (C)
RPL8	Sense	CAACCCCGAAACAAAGAAAA	62.4°
	Antisense	ATACGACCTCCACCAGCAAC	
ACTB	Sense	TGCGTGACATCAAGGAGAAG	60.9
	Antisense	AGAGCTAGAGGCAGCTGTGG	
POLR2E	Sense	ATCAACATCACGGAACACGA	60
	Antisense	GCAGCTCCGTCACTTCTTCT	
GAPDH	Sense	TATCTTCCAGGAGCGTGACC	60
	Antisense	TCTCCATGGTGGTGAAGACA	
RPS13	Sense	AAAGGCTTGACTCCCTCACA	60
	Antisense	ATGTTTGCGAACAGCAACAG	
StAR	Sense	GCCAAAGACCATCATCAACC	61.6
	Antisense	GACCAAAGCACTCAACAGCA	
CYP11A1	Sense	GGTGTCTACGAGAGCGTGAA	64.4
	Antisense	GTTGCGGTAGTCACGGTATG	
CYP17A1	Sense	GACATCTTCCCCTGGCTACA	64.4
	Antisense	CACAGTGTCCCCACAGAATG	
HSD3B1	Sense	TCTCCAGGAAGGAGGCTTTA	62.4
	Antisense	GTAGAACTGCCCCCTGATGT	
HSD17B1	Sense	GAGAGGGACCACGGTGCTGAT	64.4
	Antisense	AGTGGCGAACACTTTGAACC	
PR	Sense	CCCAGTCTCTAACGCAAAGG	65
	Antisense	GCTCAATGCCTCGTAAAACA	
ESR1	Sense	GATAATAGGCGCCACAGCAT	62.9
	Antisense	TAGTCGTTGCACACAGCACA	

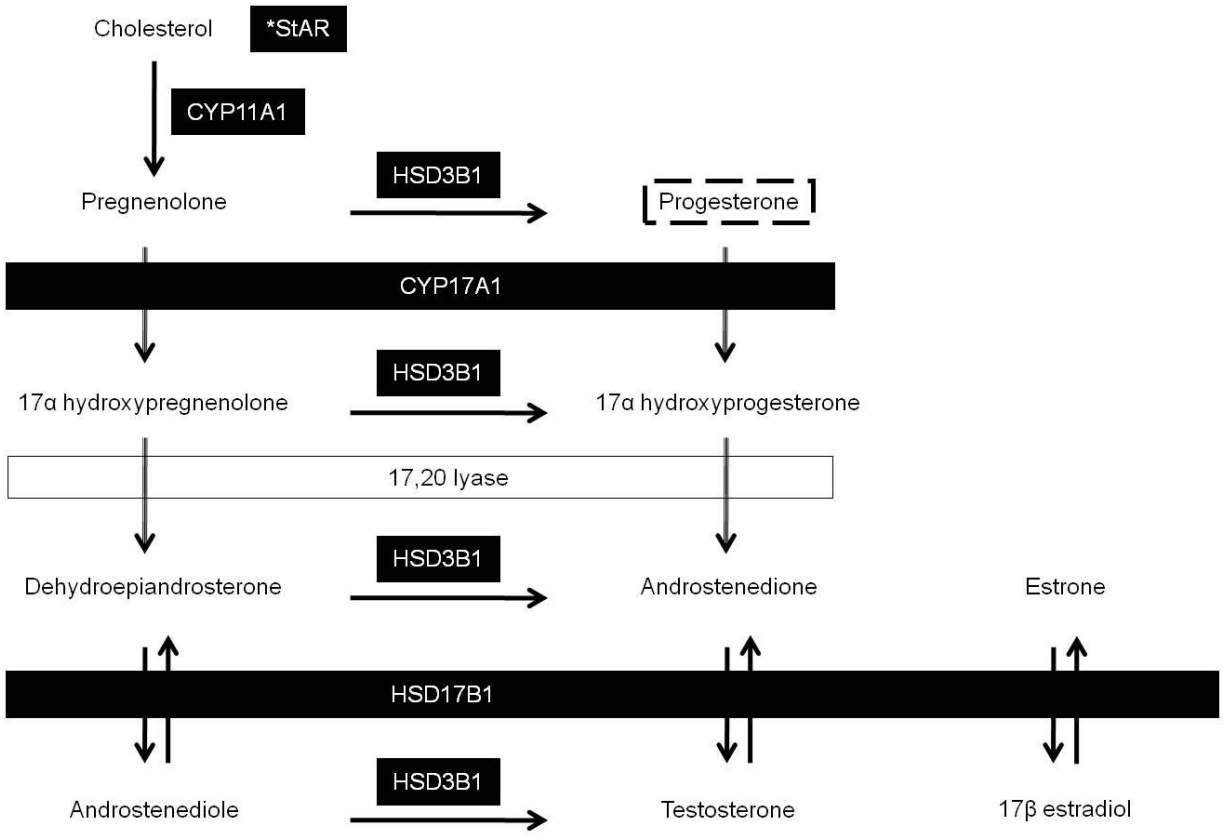


Figure 2-1. A simplified version of steroid biosynthesis highlighting the specific steroidogenic enzymes investigated. Filled boxes highlight the steroidogenic enzymes examined by RT-qPCR. Progesterone (P4) is highlighted as the focus of our study. First, the transport protein, StAR is needed to facilitate the movement of cholesterol from the outer to inner mitochondrial membrane. Cholesterol is then converted to pregnenolone by the action of CYP11A1. Pregnenolone can then be converted to either 17α-hydroxypregnenolone by CYP17A1 or to P4 by HSD3B1. P4 can either be a final product in this pathway or serve as a precursor in the synthesis of glucocorticoids, androgens, or estrogens. HSD17B1 functions in the inter-conversion of weaker and stronger androgens and estrogens and was included as a marker of upstream steroid enzyme activity [84, 97].

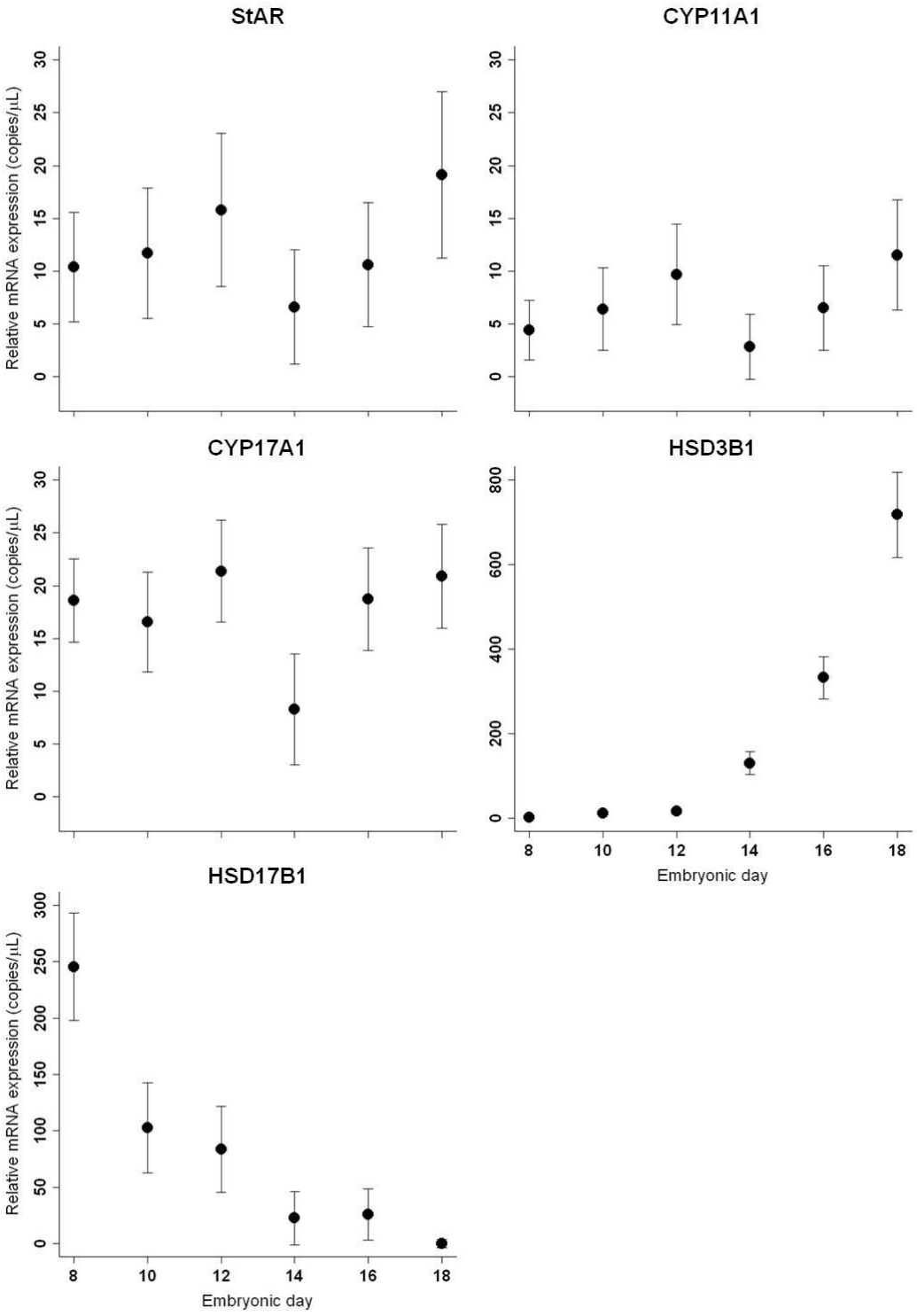


Figure 2-2. Relative mRNA expression of steroidogenic enzymes in the chick CAM. RT-qPCR analysis of mRNA coding for StAR, CYP11A1, CYP17A1, HSD3B1 and HSD17B1 on chick embryonic days 8, 10, 12, 14, 16, and 18 Data are reported as relative mRNA expression and represent mean normalized mRNA transcript number in copies/µL ± SEM.

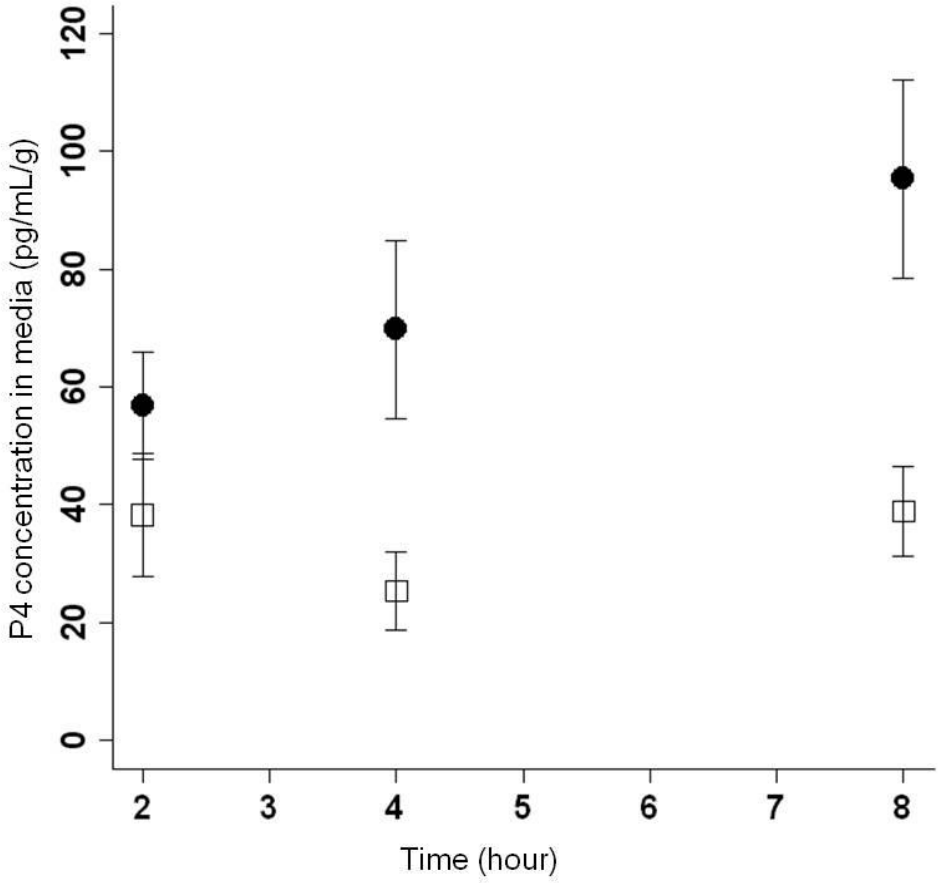


Figure 2-3. Progesterone synthesis in the chick CAM. CAM sections were incubated in culture media for 2, 4, or 8 hours either with (circles) or without (squares) cholesterol (plus cAMP) as a precursor. Concentration of P4 in the culture media is represented as pg/mL of P4 per g of CAM tissue (pg/mL/g). To determine background and cross-reactivity of the P4 assay, controls consisting of only cholesterol and cAMP were incubated for 8 hours with an average P4 concentration of 0.337pg/mL/g ± 0.423 SEM (not shown).

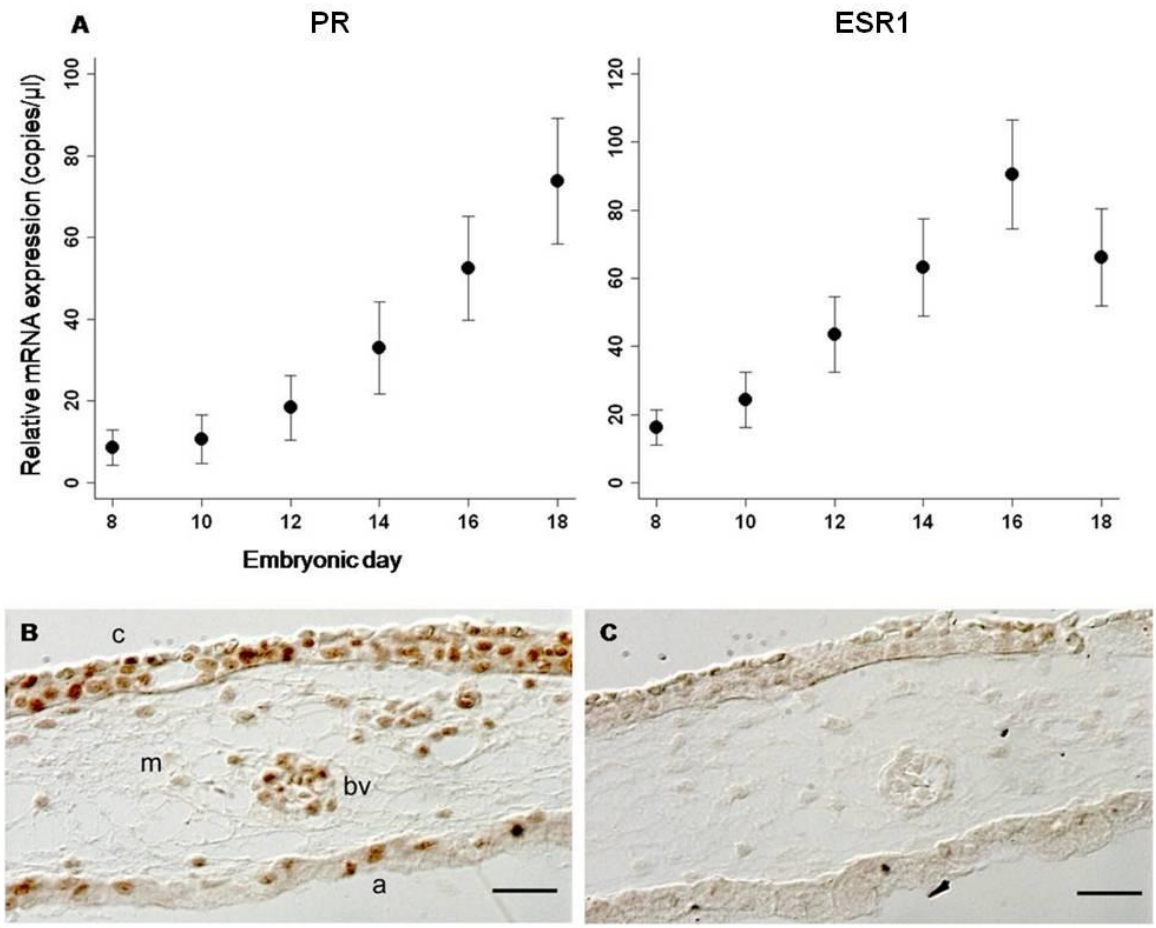


Figure 2-4. PR and ESR1 mRNA expression and PR immunolocalization in the chick CAM. (A) RT-qPCR analysis of mRNA coding for PR and ESR1 on chick embryonic. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript number in copies/µL ± SEM. (B, C**)**, PR IHC of embryonic day 18 CAM. (B) PR positive section. Nuclear staining of PR is localized predominately to the chorionic epithelium (c), and epithelial cells of blood vessels (bv). Positive nuclear staining is also present in allantoic epithelium (a), and mesenchyme (m). (C) Negative control of corresponding CAM section incubated without primary PR antibody does not show specific nuclear staining. Scale bar represents 10 microns.

CHAPTER 3
THE CHORIOALLANTOIC MEMBRANE OF THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*) HAS THE CAPABILITY TO PERFORM STEROID BIOSYNTHESIS AND RESPOND TO STEROID HORMONE SIGNALING

The mammalian placenta is an endocrine organ that functions in the synthesis, transport and metabolism of numerous hormones, including steroid hormones during pregnancy [111]. Likewise, it is well established that the ability of the mammalian placenta to perform these steroidogenic functions is crucial for the survival, development and health of the developing embryo [112, 113]. More recently, it has been suggested that disruption of normal placental steroidogenesis not only affects the embryo during pregnancy, but can also have long lasting effects into adulthood, a condition referred to as the fetal or embryonic origins of adult disease [114, 115]. While it is now understood that steroidogenesis is a critical function of the placenta, the evolution of steroidogenesis in this organ is poorly understood. We have hypothesized previously, that the ability of the placenta to perform steroidogenesis likely evolved in the extraembryonic membranes of an oviparous ancestor [36, 116].

Mammals, reptiles and birds compose the group of vertebrates known as amniotes. The formation of common extraembryonic membranes during development is a defining characteristic of this group and amniote extraembryonic membranes; amnion, allantois, chorion and yolk sac, share many structural and functional similarities across taxa due to their shared evolutionary ancestry [55]. Within amniotes, the chorioallantoic placenta evolved with the transition to viviparity (live-birth) from oviparity (egg-laying) and is a composite organ composed of a maternal and an embryonic contribution [4, 36]. The maternal contribution to the placenta consists of uterine tissues, whereas the embryonic contribution consists of extraembryonic membranes [56]. The embryonic

components to the placenta are present in all amniotes, regardless of parity mode [36]. For instance, the apposition of chorion and allantois forms the chorioallantois, and this structure either lines the inner surface of the eggshell and is referred to as the chorioallantoic membrane (CAM) in oviparous amniotes or apposes to the maternal uterus and forms the chorioallantoic placenta in viviparous amniotes [14, 43]. Likewise, the yolk sac is present in all amniotes, but will appose to the maternal uterus and form the yolk sac placenta in some, but not all viviparous amniotes [14, 43].

Prior to the early 1900s, the mammalian placenta was considered to primarily function as an organ of protection, nutrient transfer, gas exchange and waste removal. An endocrine role for the mammalian placenta was first suggested by Joseph Halban in 1905 (as cited in [6]) and for more than half a century an endocrine placenta was viewed as an exclusive trait of eutherian mammals. However, eutherian mammals are not the only amniotes that reproduce by viviparity and form a placenta. In fact, viviparity and placentation has evolved independently over 100 times within the squamate reptiles (lizards and snakes) [7, 8]. Although first described in the 1930s, the reptilian placenta, like its mammalian counterpart, was thought to act primarily in protection, nutrient transfer, gas exchange and waste removal but starting in the early 1980s, evidence of an endocrine role for the squamate placenta was obtained (for reviews, see [14, 36, 49]. Consequently, a steroidogenic placenta has now been documented in three species of viviparous lizards [21, 32, 77] indicating that a steroidogenic placenta is not an innovation of eutherian mammals [36].

Steroid biosynthesis is a common characteristic of the mammalian placenta; however, the steroidogenic enzymes expressed and thus, the hormones synthesized by

the placenta varies among species [111]. That is, in many species the chorioallantoic placenta does not express all of the enzymes of the sex or stress steroid synthesis pathways. Steroid biosynthesis begins with cholesterol and proceeds by the conversion of one steroid hormone to another by the action of specific enzymes [84]. Progesterone (P4) biosynthesis is dependent on the steroidogenic enzyme, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1), which converts pregnenolone (P5) to P4 [97]. Androgen biosynthesis requires cytochrome P450, family 17, subfamily A, polypeptide 1 (CYP17A1) to convert P5 or P4 to dehydroepiandrosterone or androstenedione (A), respectively [97]. The biosynthesis of estrogens is dependent on the enzyme, cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1) to convert A or testosterone (T) to estrone or estradiol 17β (E2), respectively [97]. Despite species differences in the levels and timing of expression, most eutherian placentae also appear to be targets of the major classes of sex and stress hormones and typically exhibit expression of the nuclear steroid receptors, such as the progesterone receptor (PR) [117], androgen receptor (AR) [118], glucocorticoid receptor (GR) [119], estrogen receptor α (ESR1) [120] and estrogen receptor β (ESR2) [121].

Currently, the extraembryonic membranes of oviparous amniotes are believed to function in the same primary roles that the placenta was assumed to perform before an endocrine role was discovered; i.e. protection, nutrient transfer, gas exchange and waste removal. Yet, it has now been established that both the maternal tissues and the extraembryonic membranes contribute to steroidogenesis in mammalian [71, 78, 79] and lizard [21] placentas. Therefore, one could hypothesize that the extraembryonic

membranes of oviparous amniotes, sharing a conserved evolutionary history, could also perform steroidogenesis. Recently, our laboratory reported that the extraembryonic membranes of one oviparous amniote, the CAM of the chicken (*Gallus gallus*), has the capability to synthesize and respond to the signaling of P4 ([116], Chapter 2). We demonstrated that the chicken CAM exhibited mRNA expression of steroidogenic enzymes involved in P4 biosynthesis, was capable of *in vitro* P4 synthesis, and exhibited mRNA and protein expression of the PR. This indicated that steroidogenic extraembryonic membranes are not an exclusive characteristic of viviparous amniotes and we hypothesized that steroidogenic extraembryonic membranes might be an evolutionarily conserved characteristic of all amniotes ([116], Chapter 2).

Here, I investigated our hypothesis by examining steroidogenesis in the CAM of another oviparous archosaurian amniote, the American Alligator (*Alligator mississippiensis*). If the alligator CAM is capable of steroidogenesis, then it should express the enzymes required for steroid hormone biosynthesis and the receptors required to respond to steroid hormone signaling. First, I investigated whether the alligator CAM demonstrates expression of mRNA coding for the nuclear receptor 5A1 (NR5A1, also named steroidogenic factor-1), a key regulator of steroidogenesis [122] and HSD3B1, CYP17A1 and CYP19A1, the key steroidogenic enzymes involved in the synthesis of progestins, androgens and estrogens; respectively [97]. Second, I investigated if the alligator CAM demonstrates expression of mRNA coding for PR, AR, GR, ESR1 and ESR2, the key receptors responding to the signaling of progestins, androgens, glucocorticoids and estrogens, respectively [123]. In addition, I examined protein immunolocalization to determine if steroid receptor mRNA is translated to a

functional protein in the case of PR and ESR1. Further, I examined whether steroidogenic factor, enzyme and steroid receptor mRNA are expressed at the same levels in the alligator CAM or if there are differences in the relative levels of gene expression.

In crocodilians, sex is determined by incubation temperature during a critical window of embryonic development, referred to as the thermo-sensitive period (TSP). In the American alligator, the TSP occurs during the embryonic stages 21 to 24 [124] and eggs incubated at 33.0 to 33.5°C result in the development of a male gonad; whereas, eggs incubated at 30°C result in the development of female gonad [124, 125]. In the placenta, gene expression has been observed to change during the course of pregnancy [126, 127] and to be differentially expressed between male and female fetuses [128]. Therefore, I examined whether steroidogenic factor, enzyme and steroid receptor mRNA expression in the CAM changes between the: 1) three embryonic stages corresponding to the stages before, during and after the TSP of sex determination, or 2) incubation temperatures which give rise to male or female embryos.

Materials and Methods

Egg Collection and Sample Preparation

Fieldwork was conducted under permits from the Florida Fish and Wildlife Conservation Commission (#WX01310h). During the summer of 2007, 4 clutches of American alligator eggs were collected from nests located within Lake Woodruff National Wildlife Refuge (Deland, Florida) and were transported to the University of Florida for incubation. Within 48 hours of arrival, eggs were candled to assess viability and embryos from 2 to 3 eggs per clutch were used to determine the average embryonic stage of the clutch based on criteria described by Ferguson [129]. Eggs from

each clutch were then evenly distributed for incubation either at a female producing temperature (FPT) of 30°C or a male producing temperature (MPT) of 33°C. At embryonic stages 19, 23 and 25, CAMs were dissected away from the embryo and eggshell, washed in 1X phosphate buffered saline (PBS) and preserved for subsequent analysis.

RNA Isolation and Reverse Transcription

Dissected CAM was stored in the RNA preservative, RNAlater solution (Ambion) at 4°C overnight and stored at -20°C until RNA isolation. Total RNA was isolated from CAM with TRIzol reagent (Invitrogen Life Technologies), purified with the SV Total RNA Isolation System (Promega), and reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). Total RNA was treated with ribonuclease-free deoxyribonuclease I (DNase I; Qiagen) to remove any genomic DNA contamination. Concentrations and quality of RNA samples were evaluated by measuring optical density with a NanoDrop ND-1000 (Thermo Scientific) and by formaldehyde gel electrophoresis. One µg of total RNA was reverse transcribed in 20 µL reaction and complementary DNA (cDNA) was stored at -20°C until RT-qPCR analysis.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR analysis was performed on CAM samples from eggs incubated at either the FPT or MPT at embryonic stages 19 (FPT n=8, MPT n=8), 23 (FPT n=8, MPT=10) and 25 (FPT n=12, MPT n=9). Complementary DNA was analyzed in triplicate by RT-qPCR amplification using an iCycler MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). Each 15-µL DNA amplification reaction contained 10 mM Tris-HCl (pH 7.84), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dNTPs, 0.5% Tween-20, 0.8% glycerol, 2% DMSO, 200-fold dilution of SYBR Green (Invitrogen), 0.01 µM Fluorescein

Calibration Dye (Bio-Rad), 0.2 μM of each primer, 0.01 U/μL AmpliTag Gold DNA polymerase (Applied Biosystems) and 25-fold dilution of cDNA. RT-qPCR amplification conditions included an enzyme activation step of 95°C (8 min) followed by 40 cycles (internal control genes) or 50 cycles (target genes) of 95°C (15 sec) and a primer specific combined annealing/extension temperature (1 min). The specificity of amplification was confirmed by melt-curve analysis. RT-qPCR has been used in previous studies to measure mRNA expression levels of each of our target genes in the American alligator [130-134]. Primer pairs were combined and diluted to a final concentration of 10 μM. Primer sequences, annealing/extension temperatures and GenBank accession number or reference are reported in Table 3-1.

Triplicate data for each gene were averaged and mRNA expression levels of the steroidogenic factor (NR5A1), steroidogenic enzymes (CYP17A1, HSD3B1 CYP19A1) and steroid receptors (PR, AR, GR, ESR1, ESR2) were determined by the absolute quantification method [87]. In brief, copy numbers were calculated from the cycle threshold (Ct) value by the linear regression of an absolute standard curve. Absolute standard curves for each target gene were generated from a plasmid containing the amplicon of interest at known concentrations as previously reported in ([116], Chapter 2) (see cloning section below). Controls lacking cDNA template were included on every RT-qPCR plate to determine the specificity of target cDNA. Additionally, to confirm that target cDNA was not contaminated by genomic DNA, RT-qPCR was performed with RPL8 primers on the RNA isolated from every sample.

RT-qPCR Normalization

To normalize mRNA expression levels, RT-qPCR was performed on all samples with three internal control genes: actin, beta (ACTB), 18S ribosomal RNA (RN18S1),

and ribosomal protein L8 (RPL8). For comparisons of embryonic stage effects within an incubation temperature, all samples were normalized using RN18S1 expression. Data are reported as relative expression and represent mean normalized mRNA transcript percentage in (% of RN18S1). For comparisons of incubation temperature effects within each embryonic stage, all samples were normalized using ACTB expression. Data are reported as relative expression and represent mean normalized mRNA transcript percentage in (% of ACTB).

Cloning and Sequencing of Plasmids

RT-qPCR of pooled cDNA was used to generate a PCR product for each primer set and amplified PCR products were separated on a 2% agarose gel and visualized by ethidium bromide on a Gel Doc EQ with Quantity One 4.6 software (Bio Rad). RT-qPCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega) and purified samples were confirmed by electrophoresis on a 2% agarose gel. PCR products were cloned into a pGEM®-T Vector System (Promega). Plasmid DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega) and sequenced on an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems) using a BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). The specificity of cloned DNA was confirmed using BLAST against sequences available in Genbank. Clone DNA concentration was quantified by NanoDrop™ ND-1000, converted to copies/µL, and serially diluted in a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 5 µg/mL of tRNA.

Immunohistochemistry and Microscopy

Dissected CAM was fixed in 4% paraformaldehyde at 4°C overnight. Tissues were washed in 1X PBS and stored in 75% ethanol at 4°C. CAMs were dehydrated,

paraffin embedded and sectioned at 8 microns. Tissue sections were deparaffinized in citrosolv and rehydrated through graded concentrations of ethanol to dionized water. Immunohistochemistry was performed using the Vectastain® Universal Quick Kit, R.T.U. (Vector Laboratories) with the following modifications: for antigen retrieval, slides were autoclaved for 30 min in 10 mM sodium citrate buffer (pH 6.0). Sections were treated with 3% hydrogen peroxide for 20 min at room temperature, blocked in normal horse serum (NHS) for 1h, and treated with the Avidin Biotin blocking kit (Vector Laboratories). Between all incubation steps, slides were washed in 0.1 M Tris buffered saline (TBS, pH 7.6) (5 min), 0.1 M TBS containing 0.2% Tween 20 (5 min), and again in TBS (5 min). CAM sections from stage 19 (n=3), stage 23 (n=2) and stage 25 (n=3) were incubated at a 1:50 dilution of mouse monoclonal anti- progesterone receptor antibody (Ab-8) or a 1:1000 dilution of mouse monoclonal anti-estrogen receptor (Ab-10), Thermo Scientific. Sections were treated with 3, 39-diaminobenzidine (Vector Laboratories) for 1.5 min for PR or 30 sec for ESR1 and washed in running tap water for 5 min. A control section receiving normal horse serum in place of primary antibody was included on every slide. Slides were dehydrated, cleared and mounted with Permount™ mounting media (Fisher Scientific). Sections were imaged using an Olympus BX60 microscope under differential inference contrast (DIC) and Zeiss AxioCAM MR3 camera with Zeiss AxioVision LE 4.8.2 software.

Statistical Analysis

All statistical analyses were performed in the R statistical programming environment version 2.13.1 [95]. For gene expression analyses, the total numbers of mRNA transcripts for 3 internal control and 9 target genes from the CAM were determined by RT-qPCR. For internal control gene analysis, I used linear mixed effects

models (LMMs) to estimate mRNA expression. In these analyses embryonic stage and incubation temperature were fixed effects on mRNA expression and RT-qPCR plate was treated as a random effect. Model assumptions were evaluated visually via examination of residuals and QQ plots and log or square-root transformations were performed when necessary to normalize errors.

To quantify relative expression of target genes, I normalized each sample to RN18S1 expression for comparisons of embryonic stage effects within an incubation temperature and to ACTB expression for comparisons of incubation temperature effects within each embryonic stage. I used two different normalization genes for these analyses because I was unable to isolate a single control gene that was unaffected by both incubation temperature and embryonic stage, see results section for further explanation. I used LMMs to compare relative mRNA expression for the three embryonic stages. In these analyses, embryonic stage was treated as a fixed effect, and RT-qPCR plate and maternal clutch were treated as random effects. Inclusion of maternal clutch; however, did not significantly reduce model deviance and was excluded from the model for all genes except NR5A1 and CYP17A1 at the FPT. Model assumptions were evaluated visually via examination of residuals and QQ plots and log or square-root transformations were performed when necessary to normalize errors. Outliers were identified from residuals and QQ plots and removed from the study (note that inclusion of outliers did not change patterns of inference, but were excluded from the final analysis because they have a disproportionate influence on mean estimates and caused violations of normality). The assumption of homogeneity of variances was met for all genes. Two samples were lost in preparation for the RT-qPCR run of NR5A1

and account for the difference in the number of starting samples for NR5A1 as compared to the other genes.

To analyze each target gene for incubation temperature effects within an embryonic stage, I used unpaired, two-sided Student's t-test to compare means from CAMs incubated at the FPT to means from CAMs incubated at the MPT within each embryonic stage. Assumptions of normality were evaluated with a two-sided F-test and log transformations were performed when necessary to normalize errors. In the event that error structure could not be normalized by transformation as was the case in ESR1 at embryonic stage 23, the Welch (Satterthwaite) approximation for the effective degrees of freedom was used.

Results

Internal Control Expression among Embryonic Stages and between Incubation Temperatures

A significant change was observed in each of our internal control genes associated with developmental conditions, either among embryonic stages or between incubation temperatures. I found that both ACTB and RPL8 expression was different among embryonic stages, but neither ACTB nor RPL8 were differentially expressed between incubation temperatures (Figure 3-1). In contrast, RN18S1 expression was not different among embryonic stages, but was differentially expressed between the two incubation temperatures (Figure 3-1). In an attempt to correct for this problem, the geometric mean was calculated according to geNORM [88], which generated a normalization factor (NF) for each sample. Unfortunately, the NF was also different among embryonic stages as well as between incubation temperatures (Figure 3-1). Due to these limitations, I was unable to perform multiple comparisons between embryonic

stages and incubation temperatures and was confined to making comparisons of incubation temperature effects within each embryonic stage and embryonic stage effects within each incubation temperature.

Steroidogenic Factor, Enzyme and Steroid Receptor Expression among Embryonic Stages

I identified for the first time, the presence of steroidogenic factor and enzyme mRNAs in the CAM of an oviparous reptile. At both the female and male producing temperatures, mRNA expression levels of steroidogenic factor and enzymes exhibited the general pattern NR5A1 > HSD3B1 ≈ CYP17A1 > CYP19A1. At all three stages, NR5A1 was the highest expressed factor and CYP19A1 was the lowest expressed enzyme, whereas HSD3B1 and CYP17A1 were expressed at similar relative levels (Figure 3-2, Figure 3-3).

The effect of embryonic stage on the level of steroidogenic factor and enzyme mRNA in the alligator CAM was examined at the two incubation temperatures. At both incubation temperatures, there was significant mRNA expression of the steroidogenic factor and enzymes (Table 3-2). At the female producing temperature (FPT), the level of expression did not change significantly among embryonic stages for CYP19A1 (Figure 3-2). However, mRNA expression of NR5A1 decreased 2.6 fold, HSD3B1 decreased 7.5 fold and CYP17A1 decreased 6.5 fold between embryonic stages 19 and 25 (Figure 3-2). At the male producing temperature (MPT), mRNA expression of NR5A1 decreased 2.4 fold, HSD3B1 decreased 9 fold and CYP17A1 decreased 4.5 fold between embryonic stage 19 and 25 (Figure 3-3). In contrast, CYP19A1 expression increased 1.5 fold between embryonic stage 19 and 25 (Figure 3-3).

Additionally, I show for the first time the presence of steroid receptor mRNAs in the CAM of an oviparous reptile. At both incubation temperatures and all three embryonic stages, the mRNA expression levels of steroid receptors exhibited the pattern GR > ESR1 > PR > AR > ESR2 (Figure 3-4, Figure 3-5). GR was the most highly expressed steroid receptor and ESR2 was the lowest expressed receptor, ESR1 exhibited the second highest level of relative expression and was expressed at approximately an order of magnitude higher than PR and AR, which had similar relative levels of expression, but with PR > AR (Figure 3-4, Figure 3-5).

At both incubation temperatures there was significant expression of steroid receptor mRNA (Table 3-2). At the FPT, the level of expression did not change significantly among embryonic stages for PR (Figure 3-4); however, mRNA expression of AR decreased 3.8 fold and GR and ESR1 both decreased 2 fold between embryonic stage 19 and 25; whereas, ESR2 expression increased 12.3 fold between embryonic stage 19 and 25 (Figure 3-4). At the MPT, the expression of ESR1 did not change significantly among embryonic stages (Figure 3-5). However, the expression of PR decreased 1.8 fold, AR decreased 4 fold and GR decreased 2.8 fold between embryonic stage 19 and 25; whereas, ESR2 increased 2 fold between embryonic stages 19 and 23 (Figure 3-5).

Steroidogenic Factor, Enzyme and Steroid Receptor Expression between Incubation Temperatures

Next, I examined the effect of incubation temperature on the level of mRNA expression at three embryonic stages. At embryonic stage 19, the expression of CYP17A1 and CYP19A1 was not different between CAMs incubated at the FPT or MPT (Table 3-3). However, NR5A1 expression was 1.5 fold greater and HSD3B1 expression

was 2.8 fold greater in CAMs incubated at the MPT than at the FPT (Table, 3-3, Figure 3-6). Additionally at stage 19, the expression of AR, GR and ESR1 was not different between CAMs incubated at the FPT or MPT (Table 3-3), whereas PR expression was 1.3 fold greater and ESR2 expression was 4 fold greater in CAMs incubated at the MPT than at the FPT (Table, 3-3, Figure 3-6).

At embryonic stage 23, the expression of steroidogenic factor and enzyme mRNA was not different between CAMs incubated at the FPT or the MPT (Table 3-3). Likewise, the expression of PR, AR and ESR2 was not different between the incubation temperatures (Table 3-3). However, GR expression was 1.4 fold greater and ESR1 expression was 1.9 fold greater in CAMs incubated at the FPT than the MPT (Table 3-3, Figure 3-7).

Finally, at embryonic stage 25, expression of steroidogenic factor and enzymes, as well as the expression of steroid receptor mRNAs was not different between CAMs incubated at the FPT or the MPT (Table 3-3).

PR and ESR1 Immunolocalization

For two of the steroid receptors, I sought to determine if PR and ESR1 mRNA was translated to protein. I performed immunolocalization of the nuclear PR and ESR1 and observed that both PR (Figure 3-8) and ESR1 (Figure 3-9) were predominately localized to the nucleus in the chorionic and allantoic epithelium and in the epithelial cells of mesenchymal blood vessels. Positive nuclear staining of both PR and ESR1 was also present to a lesser degree in the mesenchyme.

Discussion

All amniote embryos form common extraembryonic membranes during development, thus studies of steroidogenesis in the chorioallantoic membrane from

oviparous vertebrates can increase our understanding of how the chorioallantoic placenta evolved as an endocrine organ. Albergotti et al. ([116], Chapter 2) were the first to evaluate the presence of steroidogenic enzymes in the CAM of an oviparous amniote and found that the chicken CAM exhibited mRNA expression of steroidogenic acute regulatory protein (StAR) ,cytochrome P450 11A1 (CYP11A1), HSD3B1, CYP17A1 and hydroxysteroid (17-beta) dehydrogenase. Like the chicken, the alligator CAM exhibited mRNA expression of HSD3B1 and CYP17A1. In addition, I have shown the expression of mRNA coding for CYP19A1 and the steroidogenic factor, NR5A1, for the first time in the CAM of an oviparous amniote. The presence of steroidogenic factor and enzyme mRNAs supports our hypothesis that the alligator CAM has the potential at the molecular level to regulate and synthesize steroid hormones. NR5A1, belongs to the steroid receptor family of transcription factors and is considered a master regulator of steroidogenesis [122]. In the alligator CAM, NR5A1 was more highly expressed than any of the steroidogenic enzymes examined, suggesting a role for NR5A1 in the regulation of steroidogenesis in this tissue. HSD3B1 and CYP17A1 were expressed at greater relative levels than CYP19A1, possibly suggesting that the CAM synthesizes progestins and androgens at a higher level than estrogens.

In eutherian placentae, it is typical to see differences in the steroidogenic factors and enzymes expressed and species-related differences are evident in the expression of HSD3B1, CYP17A1 and CYP19A1 as well as the corresponding hormones synthesized [111]. HSD3B expression and the ability to synthesize P4 appear to be a fundamental characteristic of steroidogenic placentae (except canine) [127, 135-137], although the timing and level of expression varies and species differences are evident in

the isoforms of HSD3B expressed in the placenta [97]. CYP17A1 expression and androgen biosynthesis is present in the placenta of some species, such as rat, pig, sheep and cow [126, 135, 136, 138, 139]; but is lacking in the horse, baboon and human placenta, requiring these species to utilize a non-placental source of androgens for placental synthesis of estrogens [72, 111, 140]. Expression of CYP19A1 and the ability to synthesize estrogens is found in the placenta of human, horse, cow, pig and sheep; however, CYP19A1 and estrogen biosynthesis is lacking in the mouse and rat placenta [135, 139, 141, 142]. Given that eutherian placentae vary in how steroidogenesis is accomplished and what steroid hormones are synthesized, it is not surprising that the alligator CAM would also reflect differences in the relative levels of steroidogenic enzymes expressed and the potential steroid hormones synthesized.

Albergotti et al.([116], Chapter 2) reported the presence of steroid receptors in the CAM of an oviparous amniote; that is, they found that the chicken CAM exhibited both mRNA and protein expression of the PR and mRNA expression of ESR1. Like the chicken, PR and ESR1 mRNAs were present in the alligator CAM and I have also shown mRNA expression of AR, GR and ESR2 for the first time in an oviparous CAM, which supports our hypothesis that the oviparous CAM has the molecular capability to respond to steroid hormone signaling. In addition, I have demonstrated that mRNA is translated to the protein level for at least two of the steroid receptors examined, PR and ESR1.

I observed that GR was the most highly expressed steroid receptor with average expression an order of magnitude higher than ESR1. ESR1, which demonstrated the second highest level of expression was an order of magnitude higher than PR and AR,

which had similar levels of relative expression, but with PR > AR. ESR2 was also present in the alligator CAM, but was expressed at relatively low levels. The levels of steroid receptor expression found here could reflect the relative importance of the alligator CAM to be able to respond to glucocorticoids, estrogens, progestins and androgens, respectively. However, it is well established that transcription does not always reflect translation to functional protein. As with the steroidogenic factor and enzymes reported above, future work has to examine the translation of mRNAs into active proteins before we can establish function or signal reception in the CAM tissues of the American alligator. Unfortunately, there is very little known about how the relative levels of steroidogenic factor, enzyme and steroid receptor mRNA compare to one another in the placenta. Many studies have identified the presence of or quantified the expression of one or two of these factors in a single study, but to the best of my knowledge, a comparison of the relative levels of mRNA as undertaken here in the CAM has not been conducted in placental tissues.

In the placenta, GR in conjunction with the placental enzyme, 11β-hydroxysteroid dehydrogenase (11β HSD), which catalyzes the interconversion of biologically active and inactive glucocorticoids, are important regulators of glucocorticoid action [119]. Glucocorticoids have been shown to be influence placental gene expression [143-145], placental and fetal growth [145], placental vascularity [146] and timing of birth [147]. During pregnancy, the human placenta synthesizes increasing concentrations of E2 and P4 [117]. E2 acting through ESR1 has been suggested to promote normal placental development [120] and both ESR1 and ESR2 appear to play a role in human trophoblast differentiation [121]. E2 also plays a role in the regulation of placental P4

synthesis, which occurs in conjunction with autoregulation by P4 itself, acting through PR [117, 148]. P4 is not only instrumental in the maintenance of pregnancy [72] and timing of parturition [73], but has also been shown to promote placental [74, 75] and fetal [74] growth and is suggested to play a role in human fetoplacental vascularization [109, 110]. AR mediated actions of androgens on the placenta remain speculative at this point in time; however, recent work has demonstrated the presence of AR mRNA and protein in human placenta and also noted that AR mRNA expression is increased in placentae from preeclamptic pregnancies [118]. Currently, I can only speculate on the role of steroid hormones in the physiology of the alligator CAM. I hypothesize that steroid hormones might have similar roles as in eutherians and act to regulate gene expression, influence differentiation, development, growth and vascularization of the CAM as well as play a potential role in the maintenance of embryonic development, timing of hatch and growth of the embryo.

Placental gene expression has been observed to change with development [126, 127] and fetal sex [128]; therefore, I investigated whether steroidogenic factor, enzyme and steroid receptor mRNA expression in the CAM changes among the embryonic stages corresponding to before, during and after the TSP or the incubation temperatures which give rise to either a male or female embryo. Overall, I found that all of the genes examined changed among the embryonic stages of our study either at one or both of the incubation temperatures (Figure 3-2 through Figure 3-4) and several genes were expressed differently between incubation temperatures (Figure 3-6, Figure 3-7) suggesting that steroidogenic factor, enzyme and steroid receptor mRNA expression is affected by conditions, such as developmental stage and temperature.

With the exception of CYP19A, which increased in expression between stage 19 and 25 in CAMs incubated at the MPT and ESR2, which increased in expression between stage 19 and 25 in CAMs at both incubation temperatures, all of the genes exhibiting significant changes among embryonic stages showed decreased expression at stage 25 relative to stage 19 or 23. This general pattern of decreased gene expression at stage 25 suggests that the CAM could shut down steroidogenesis at this point in embryonic development. The staging system used for the alligator (and many other species) is based on the accumulation of morphological traits and the stage numbers used are somewhat deceiving as they imply a linear time scale. However, alligator developmental stages are not linear in terms of the embryonic days between each stage [149]. Stage 25 is late in the developmental process and since this tissue will be discarded at hatch, the CAM could shut down steroidogenesis or even processes in general ,as evident from decreased expression of ACTB and RPL8 at stage 25 (Figure 3-1).

I note that our results showing either no change in expression among embryonic stages or decreased mRNA expression at stage 25 relative to stage 19 or 23 are different than our previous report on the chicken CAM, in which expression of HSD3B1, PR and ESR1were found to increase between day 8 and 18 of development [116]. One possible explanation for this discrepancy is that our study did not survey gene expression across embryonic development in the same manner as in the chicken CAM, which examined expression on a linear time scale of days rather than by stages and spanned the days immediately following the formation of the CAM to right before hatch, making it difficult to directly compare the patterns of expression between these two studies. Despite these distinctions; however, it does appear that the chicken and

alligator CAM could differ in the developmental expression of these genes. We have limited developmental data on embryonic plasma concentrations of steroid hormones in the American alligator. However, it has been shown that plasma concentrations of P4 are highest around the TSP period (stages 21.5 to 22.5 for males; 20 to 23.5 for females), declining thereafter and remaining low for the second half of incubation [150]. This pattern is in stark contrast to embryonic plasma P4 in the chicken, which steadily increases from day 9 to day 20 [151] and could possibly account for the differences I observed between alligator and chicken CAM expression of HSD3B1 and PR.

The placenta has historically been viewed as an asexual organ; however, a growing number of studies are reporting sexually dimorphic patterns of placental gene expression between male and female fetuses (as reviewed in [152]). For example, Mao et al. (2010) [153] reported that murine placental expressions of several isoforms of HSD3B as well as ESR1 are influenced by fetal sex and maternal diet. Within the same dietary groups, ESR1 was upregulated in female placentae relative to male placentae and Hsd3b5 was upregulated in male placentae relative to adjacent female placentae [153]. At the embryonic stage immediately prior to the TSP, I observed that the alligator CAM exhibited a temperature dependent pattern of expression of NR5A1, HSD3B1, PR and ESR2 with CAMs incubated at the MPT showing higher levels of expression than those incubated at the FPT (Figure 3-6). Additionally, I found that during the TSP, the alligator CAM showed a temperature dependent pattern of expression of GR and ESR1 with CAMs incubated at the FPT showing higher levels of expression than those incubated at the MPT (Figure 3-7).

While the underlying molecular mechanisms of temperature sex determination (TSD) remain unresolved, it has been suggested that temperature can either directly or indirectly stimulate or suppress the expression of steroidogenic factors, enzymes and steroid receptors [154-157] that could play a role in TSD. Because sex has yet to be determined at embryonic stage 19 in the alligator, the temperature dependent expression that I observed at this stage could simply be a direct or indirect result of incubation temperature, i.e., the higher temperature of the MPT enhanced CAM expression of NR5A1, HSD3B1, PR and ESR2 or it could suggest that embryo physiology is different between embryos fated to develop as male or female prior to the stage of actual gonadal differentiation. Likewise, the temperature dependent expression of ESR1 and GR observed in the CAM during the period of sex determination could again simply be a result of incubation temperature or it could reflect a sexually dimorphic pattern of gene expression in this tissue, but at this time I am unable to distinguish between these possibilities.

Our study demonstrates that the oviparous alligator CAM has the molecular mechanisms in place to perform biosynthesis of progestins, androgens and estrogens. I have not demonstrated; however, that the alligator CAM has the molecular mechanisms to perform *de novo* steroid synthesis, which would require demonstration of STAR [158] and CYP11A1 [97]. Albergotti and Guillette [36] suggested a model for the evolution of endocrine function in the reptilian yolk sac and CAM in which the CAM evolved the ability to biotransform yolk steroids as well as synthesize steroid hormones *de novo*. It is well established that the yolk of oviparous amniotes is a rich source of maternally derived steroid hormones [159]. While the majority of studies have focused on the

abundance and distribution of A, T and E2 in the yolk, recent work has indicated that P4 is present in considerably greater concentrations than both E2 and T in American alligator yolk [160]. Typically, yolk steroid concentrations are initially high and then begin to decline at some point during embryonic development [99, 161, 162]. Yolk P4 concentrations are highest at oviposition and then decline through embryonic development in the red-eared slider turtle (*Trachemys scripta*) [161]. Yolk A, T and E2 concentrations are relatively stable between embryonic stage 16 to 21 in the American alligator, but then decline dramatically in subsequent stages [163]. If the CAM is indeed capable of converting maternal yolk hormones, then decreasing yolk steroids through development could result in decreased substrate available for these processes.

In addition to maternal yolk steroids, another possibility is that the CAM is utilizing hormones synthesized by the embryo. In contrast to yolk steroids, which are present at high concentrations at ovulation, the embryo doesn't begin synthesizing steroid hormones until after gonadogenesis [164, 165]. Here, I have discussed yolk and embryo as "sources of precursors" as if they were independent entities; however, the yolk sac, embryo and CAM are all connected through circulation and the more realistic picture is that there is significant interplay and transfer of chemical messengers between the "individual" compartments [36]. The fact that the oviparous CAM expresses steroidogenic enzymes, which could play a role in converting yolk steroids, raises some interesting possibilities. Currently, it is still not fully understood how steroids are mobilized from the yolk to the embryo, and how and where yolk steroids are metabolized and exert their actions [159]. Despite numerous studies documenting a decrease in yolk steroids during embryonic development, the explanation for this

phenomenon remains to be elucidated [159, 166]. Recently, Paitz et al. showed that whole egg hormone concentrations in European starlings (*Sturnus vulgaris*) decline during embryonic development and that exogenous T in the yolk was metabolized by yolk – albumen - extraembryonic membrane homogenates, supporting a hypothesis that a decrease in yolk steroid concentrations is due to metabolism rather than dilution by or leaching to the albumen [167].

I hypothesize that the CAM plays a role in the biotransformation of steroid hormones deposited in the yolk and synthesized by the embryo. Steroid metabolism in the eutherian placentae facilitates the regulation of maternal and fetal hormone environments and the placenta is commonly thought of as a “barrier” that metabolizes active hormones to inactive metabolites to prevent the diffusion of potentially harmful hormones between the mother and fetus. However, metabolism of biologically inactive steroid metabolites to their active forms is also an important feature of the placenta. For example, estrogen sulfotransferase in the placenta catalyzes the sulfoconjugation of active estrogens and renders them hormonally inactive [112, 168]; whereas, steroid sulfatase catalyzes the hydrolysis of inactive, conjugated steroids and converts them into biologically active hormones [112, 169]. It was recently reported that estrogen sulphotransferase activity is increased in the yolk and extraembryonic membranes of the red-eared slider turtle [162] during the period of incubation when yolk E2 concentrations have been observed to decline naturally [162] and radioactive labeled E2 was shown to be converted to a water-soluble metabolite [170]. From these observations, the authors suggested “that the extra-embryonic membranes of oviparous

amniotes may serve an important role in metabolizing maternal steroids, just as they do in placental mammals [167].

The chicken ([116], Chapter 2) and alligator studies suggest that at the molecular level, the oviparous CAM of archosaurs shares the ability to synthesize steroid hormones. In addition, these two studies suggest that at the molecular and protein level, the archosaurin CAM shares the ability to respond to steroid hormone signaling. Collectively, our findings support the hypothesis that steroidogenesis and steroid hormone signaling of extraembryonic membranes could be an evolutionary conserved characteristic of amniotes. However, more work is needed and future studies should attempt to investigate protein and functional levels of steroid biosynthesis and steroid hormone signaling in oviparous extraembryonic membranes.

Table 3-1. Alligator PCR primers used for RT-Quantitative real-time PCR

Gene	Direction	Sequence (5'-3')	Annealing (C)	GenBank accession number or reference
ACTB	Sense	ATGAGGCCCAAAGCAAAAGA	60	DQ421415
	Antisense	CCCAGTTGGTGACAATGCC		
RN18S1	Sense	GTCCGAAGCGTTTACTTTGA	59.2	AF173605.1
	Antisense	TCTGATCGTCTTCGAACCTC		
RPL8	Sense	GGTGTGGCTATGAATCCTGT	62.9	Katsu et al. (2004)
	Antisense	ACGACGAGCAGCAATAAGAC		
NR5A1	Sense	CAGTCTCGAATGTGAAATACCTGGA	66.4	AF180296
	Antisense	CGCGTTGGCCTTCTCCT		
CYP17A1	Sense	CCAGAAAAAGTTCACCGAGCAC	62.9	DQ007997
	Antisense	CGGCTGTTGTTGTTCTCCATG		
HSD3B1	Sense	GTGATCCCATCTGCAATGGTG	60	Milnes et al. (2008)
	Antisense	CCATCTGCCTTCAGGACATGTT		
CYP19A1	Sense	CAGCCAGTTGTGGACTTGATCA	62	AY029233
	Antisense	TTGTCCCCTTTTTCACAGGATAG		
PR	Sense	AAATCCGTAGGAAGAACTGTCCAG	67.5	AB115911
	Antisense	GACCTCCAAGGACCATTCCA		
AR	Sense	TGTGTTCAGGCCATGACAACA	66.5	AB186356
	Antisense	GCCCATTTCACCACATGCA		
GR	Sense	AAAAAACTGTCCCGCATGCC	66.5	AF525750
	Antisense	CGTTGGACTGCTGAATTCCTTT		
ESR1	Sense	AAGCTGCCCCTTCAACTTTTTA	66.5	AB115909
	Antisense	TGGACATCCTCTCCCTGCC		
ESR2	Sense	AAGACCAGGCGCAAAAGCT	65	AB115910
	Antisense	GCCACATTTCATCATTCCCAC		

Table 3-2. Statistic summary of RT-Quantitative real-time PCR on the alligator CAM at three embryonic stages.

Gene	Incubation temperature[a]	Expression of mRNA[b]	Expression among embryonic stages[b]	Expression between stages
NR5A1	FPT	$F_{1, 13}$= 4349.97, ***	$F_{2, 13}$= 6.18, **	19>25
HSD3B1	FPT	$F_{1, 22}$= 100.4, ***	$F_{2, 22}$= 8.72, **	19>25
CYP17A1	FPT	$F_{1, 14}$= 6687.67, ***	$F_{2, 14}$= 37.87, ***	19>25
CYP19A1	FPT	$F_{1, 23}$= 9679.71, ***	$F_{2, 23}$= 0.3, ns	ns
NR5A1	MPT	$F_{1, 21}$= 13533.94, ***	$F_{2, 21}$= 7.21, **	19>25
HSD3B1	MPT	$F_{1, 21}$= 176.09, ***	$F_{2, 21}$= 23.98, ***	19>25
CYP17A1	MPT	$F_{1, 21}$= 2637.19, ***	$F_{2, 21}$= 18.24, ***	19>25
CYP19A1	MPT	$F_{1, 22}$=34.34, ***	$F_{2, 22}$=3.81, *	19<25
PR	FPT	$F_{1, 23}$= 11665.37, ***	$F_{2, 23}$= 2.0, ns	ns
AR	FPT	$F_{1, 23}$= 6082.12, ***	$F_{2, 23}$= 10.76, ***	19>25
GR	FPT	$F_{1, 23}$= 7670.70, ***	$F_{2, 23}$= 7.66, **	19>25
ESR1	FPT	$F_{1, 22}$= 269.03, ***	$F_{2, 22}$= 5.55, *	19>25
ESR2	FPT	$F_{1, 22}$ =94.51, ***	$F_{2, 22}$ =8.01, **	19<25
PR	MPT	$F_{1, 22}$= 18703.15, ***	$F_{2, 22}$= 7.21, **	19>25
AR	MPT	$F_{1, 22}$= 2390.45, ***	$F_{2, 22}$= 10.75, ***	19>25
GR	MPT	$F_{1, 22}$= 41.94, ***	$F_{2, 22}$= 17.66, ***	19>25
ESR1	MPT	$F_{1, 22}$= 192.21, ***	$F_{2, 22}$= 3.56, ns	ns
ESR2	MPT	$F_{1, 22}$=7.54, **	$F_{2, 22}$=3.82, **	19<23

[a]FPT = female producing temperature; MPT = male producing temperature

[b]Data presented as F(df)=, p-value=

*** p<0.0001

** p<0.01

* p<0.05

ns=not significant

Table 3-3. Statistic summary of RT-Quantitative real-time PCR on the alligator CAM at two incubation temperatures.

Gene	Stage 19[a]	FPT vs. MPT[b]	Stage 23[a]	FPT vs. MPT[b]	Stage 25[a]
NR5A1	t(13)=2.30, *	FPT<MPT	t(16)=0.25, ns	ns	t(18)=1.23, ns
HSD3B1	t(14)=2.42, *	FPT<MPT	t(16)=1.34, ns	ns	t(19)=0.54, ns
CYP17A1	t(14)=0.43, ns	ns	t(16)=1.81, ns	ns	t(19)=1.73, ns
CYP19A1	t(14)=0.95, ns	ns	t(16)=0.66, ns	ns	t(19)=1.50, ns
PR	t(14)=2.29, *	FPT<MPT	t(16)=1.87, ns	ns	t(19)=0.17, ns
AR	t(14)=1.28, ns	ns	t(16)=0.44, ns	ns	t(19)=0.59, ns
GR	t(14)=0.30, ns	ns	t(16)=2.26, *	FPT>MPT	t(19)=1.52, ns
ESR1	t(14)=1.14, ns	ns	t(12.15)=2.85, **	FPT>MPT	t(19)=0.50, ns
ESR2	t(14)=2.60, *	FPT<MPT	t(16)=0.51, ns	ns	t(19)=1.78, ns

[a] Data presented as t(df)=, p-value=

[b] FPT = female producing temperature; MPT = male producing temperature

** p=0.01

* p<0.05

ns=not significant

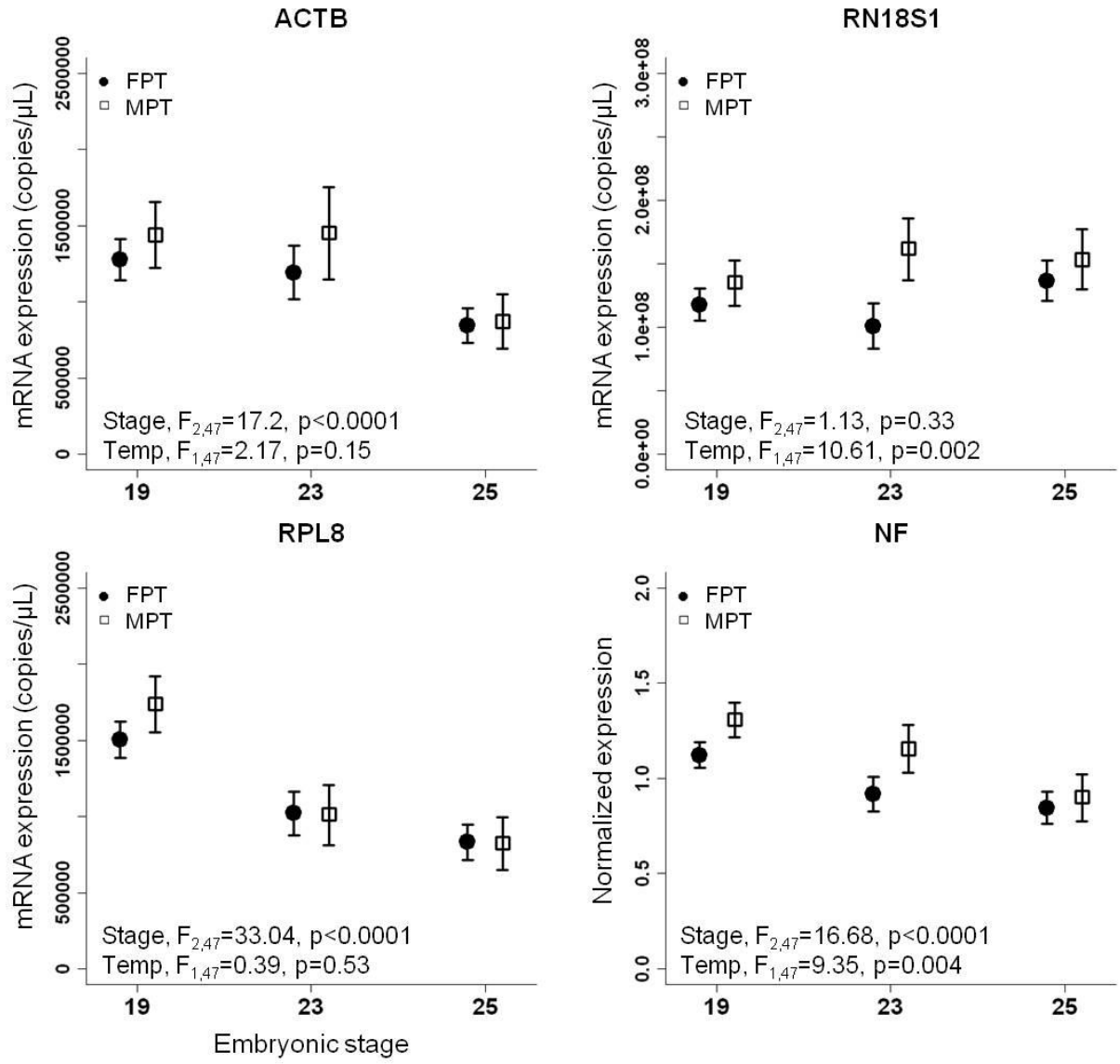


Figure 3-1. mRNA expression of internal control genes in alligator CAM. RT-qPCR analysis of mRNA coding for ACTB, RPL8, and RN18S1 at embryonic stages 19, 23 and 25 in CAMs incubated at the FPT (filled circles) or MPT (open squares). The normalization factor (NF) generated according to geNORM is also shown. ACTB, RN18S1 and RPL8 data are reported as mRNA expression and represent mean mRNA transcript number in copies/µL ± SEM. NF data are reported as normalized expression and represent the geometric mean of the three internal control genes ± SEM.

.

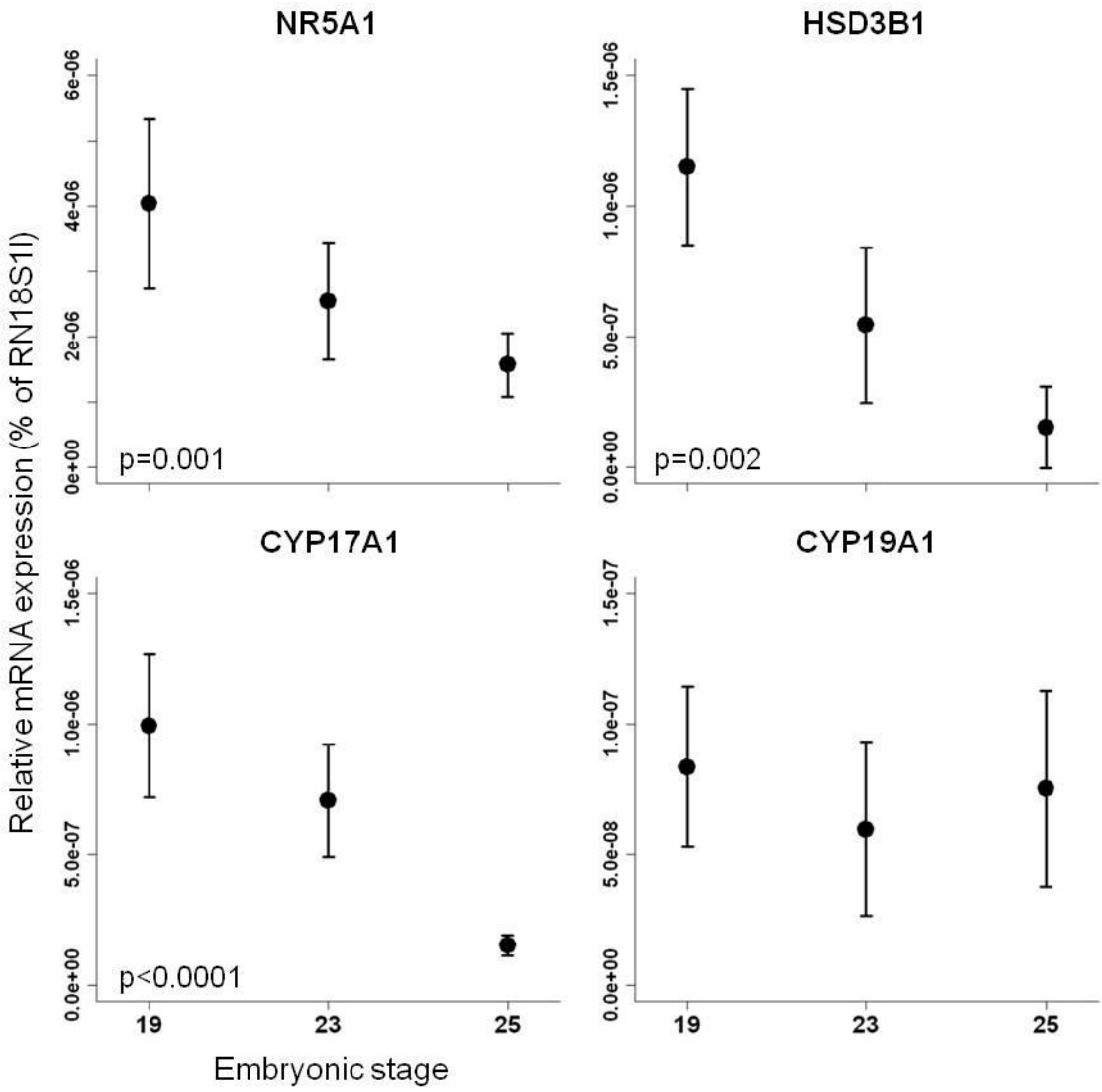


Figure 3-2. Relative mRNA expression of steroidogenic factor and enzymes in the alligator CAM incubated at the FPT. RT-qPCR analysis of mRNA coding for NR5A1, HSD3B1, CYP17A1, and CYP19A1at embryonic stages 19, 23, and 25. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RN18S1) ± SEM.

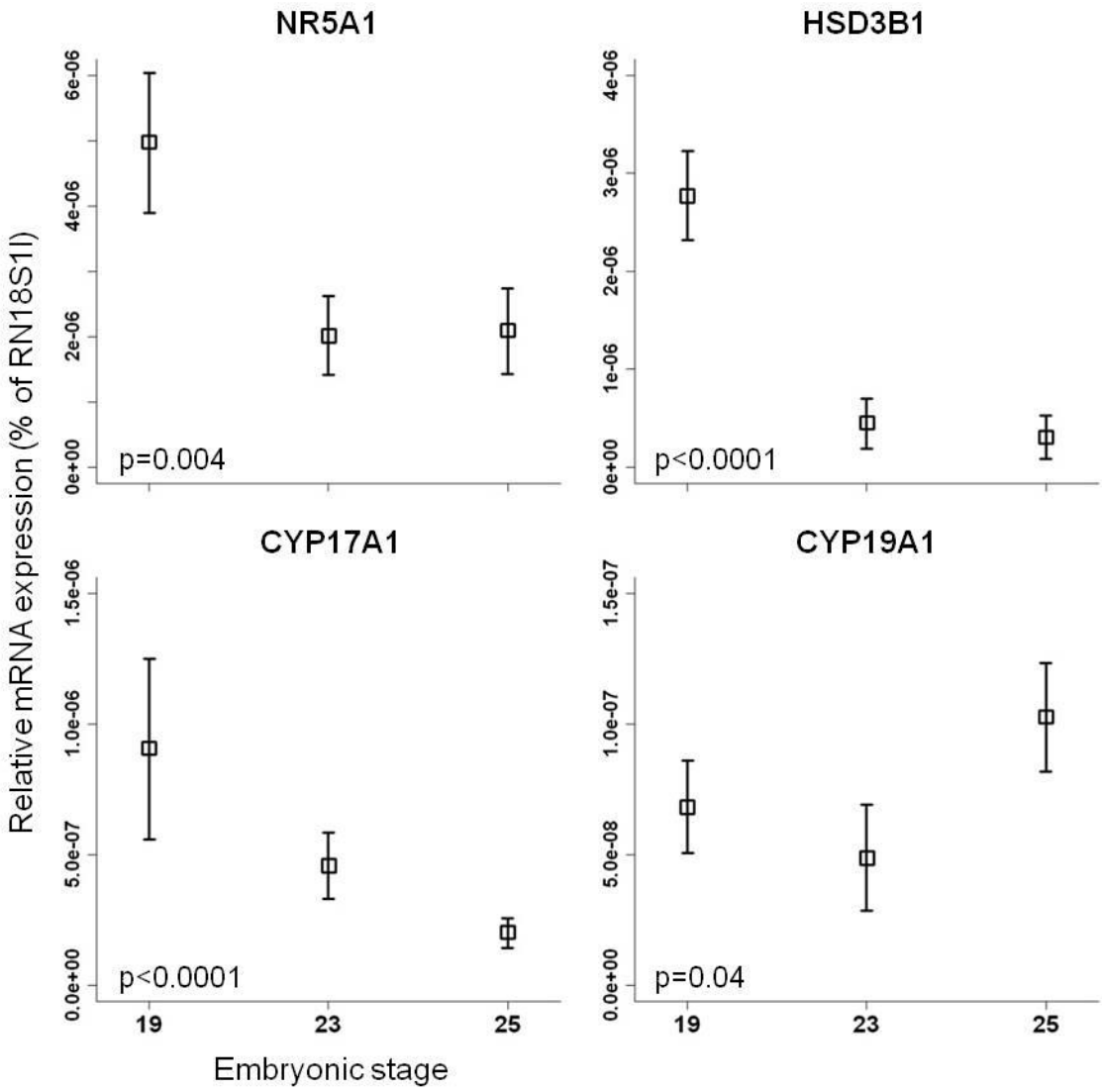


Figure 3-3. Relative mRNA expression of steroidogenic factor and enzymes in the alligator CAM incubated at the MPT. RT-qPCR analysis of mRNA coding for NR5A1, HSD3B1, CYP17A1, and CYP19A1at embryonic stages 19, 23, and 25. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RN18S1) ± SEM.

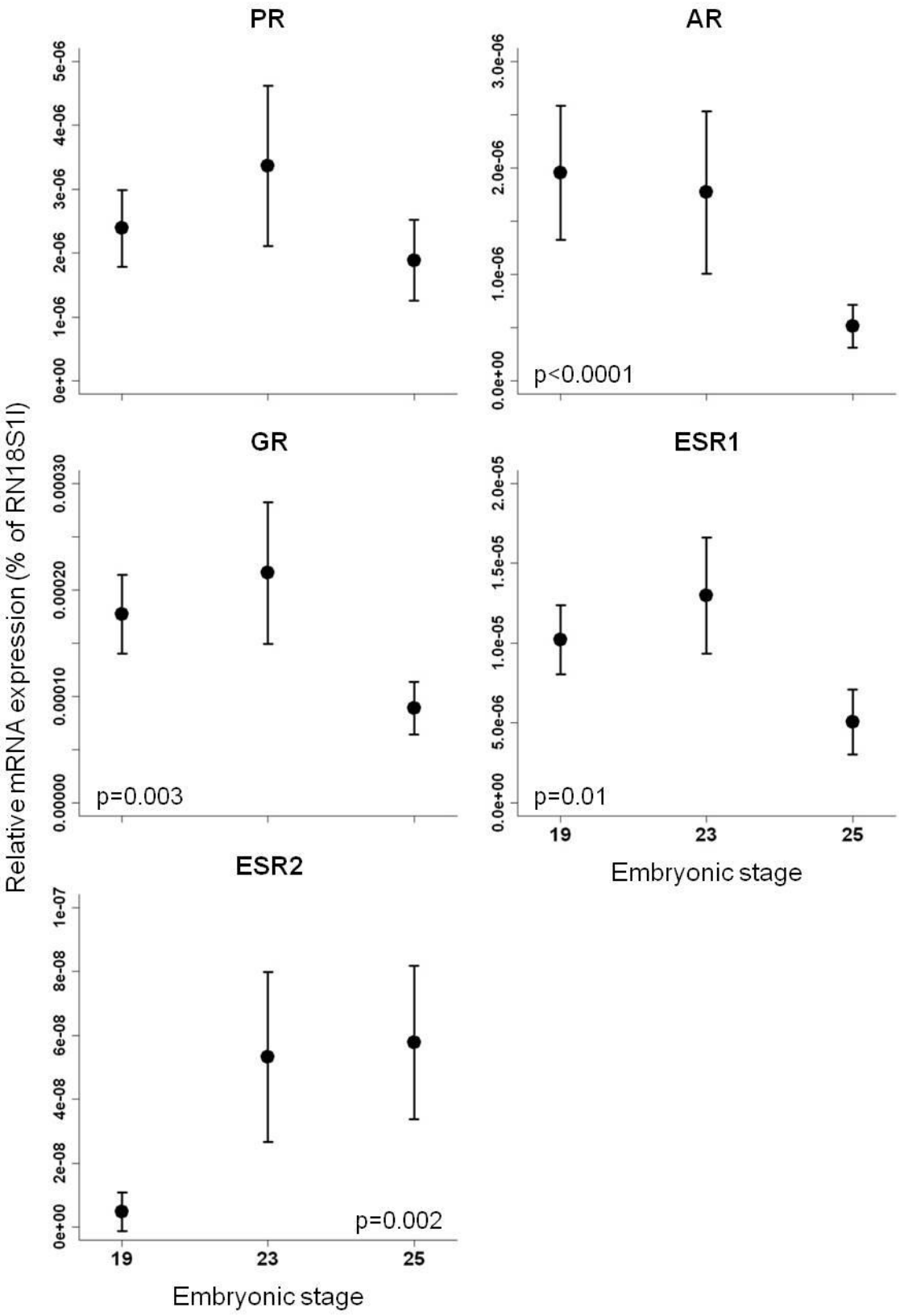

PR
AR
GR
ESR1
ESR2
Relative mRNA expression (% of RN18S1I)
p<0.0001
p=0.003
p=0.01
p=0.002
19
23
25
Embryonic stage

Figure 3-4. Relative mRNA expression of steroid receptors in the alligator CAM incubated at the FPT. RT-qPCR analysis of mRNA coding for PR, AR, GR, ESR1, and ESR2 at embryonic stages 19, 23, and 25. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RN18S1) ± SEM.

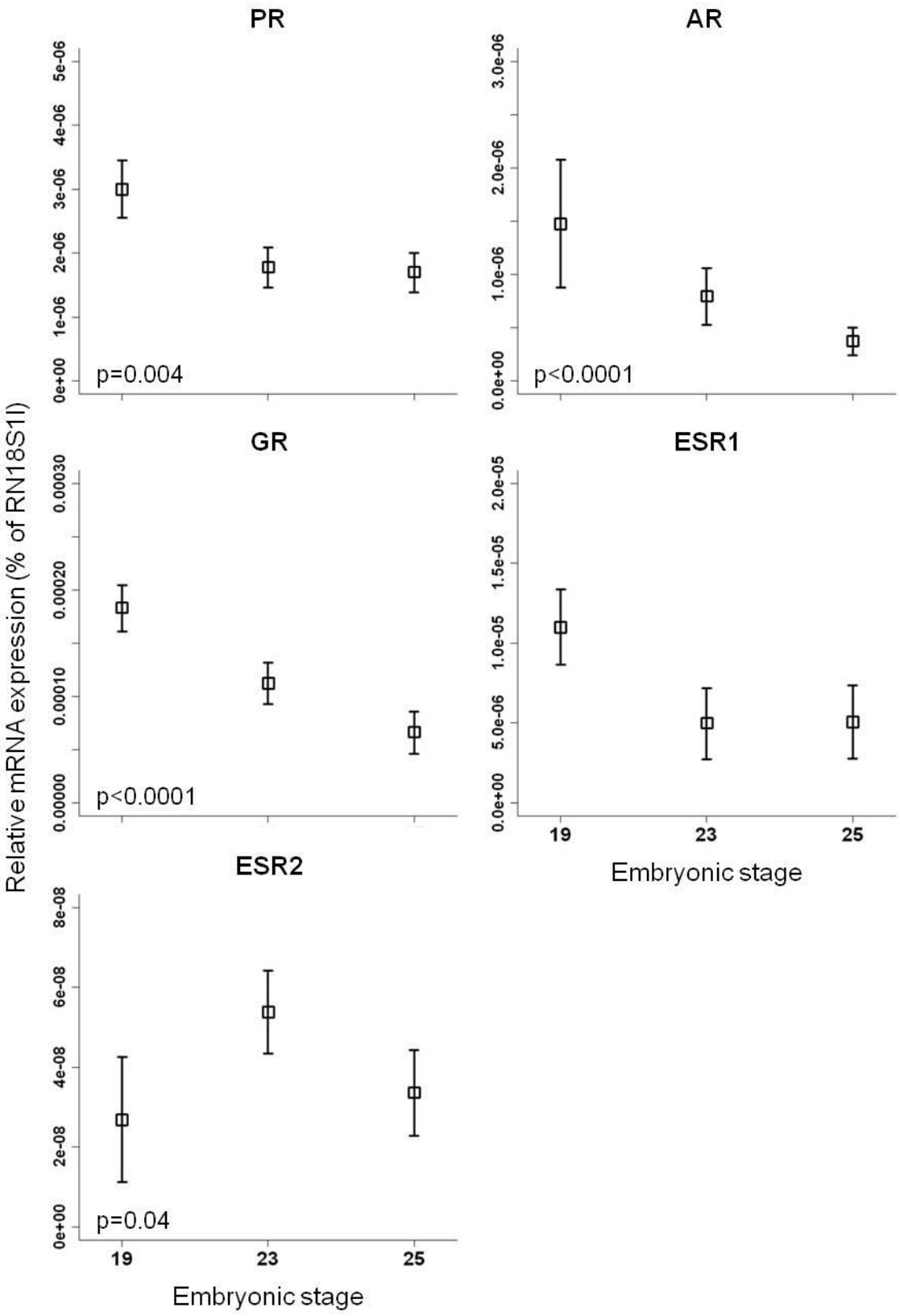

PR
AR
GR
ESR1
ESR2
p=0.004
p<0.0001
p<0.0001
p=0.04
Relative mRNA expression (% of RN18S1I)
Embryonic stage
Embryonic stage
19
23
25
0e+00
1e-06
2e-06
3e-06
4e-06
5e-06
0.0e+00
1.0e-06
2.0e-06
3.0e-06
0.00000
0.00010
0.00020
0.00030
0.0e+00
5.0e-06
1.0e-05
1.5e-05
2.0e-05
0e+00
2e-08
4e-08
6e-08
8e-08

Figure 3-5. Relative mRNA expression of steroid receptors in the alligator CAM incubated at the MPT. RT-qPCR analysis of mRNA coding for PR, AR, GR, ESR1, and ESR2 at embryonic stages 19, 23, and 25. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RN18S1) ± SEM.

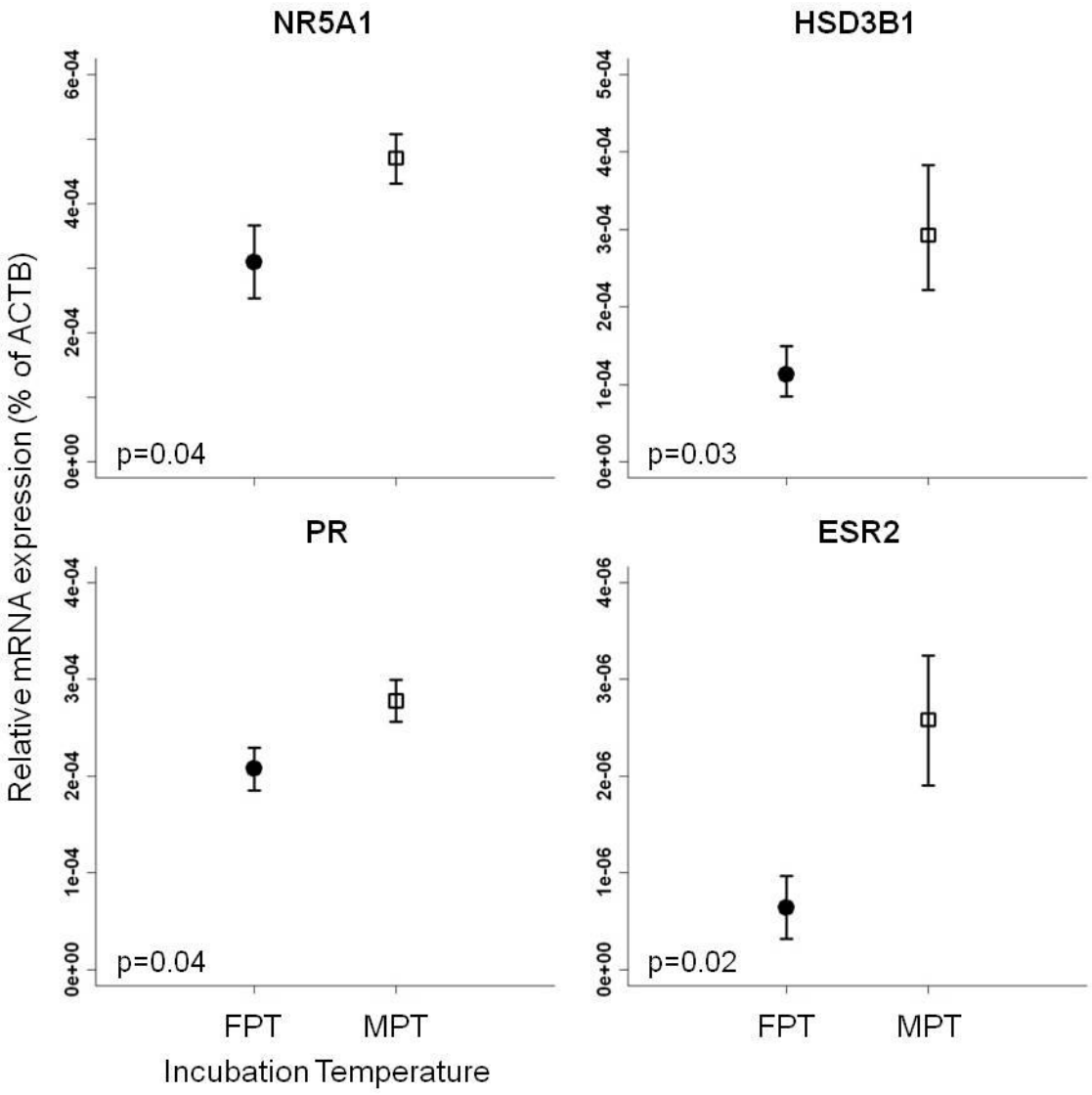


Figure 3-6. Relative mRNA expression of NR5A1, HSD3B1, PR, and ESR2 in the alligator CAM between incubation temperatures at embryonic stage 19. FPT data are represented as filled circles and MPT data are represented as open squares. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of ACTB) ± SEM.

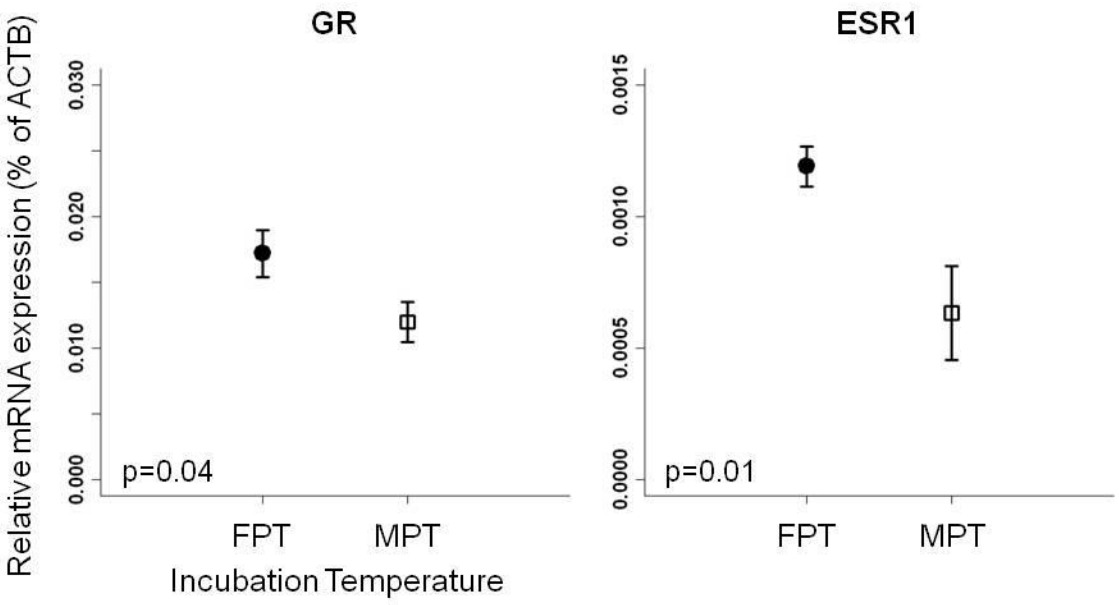


Figure 3-7. Relative mRNA expression of GR and ESR1 in the alligator CAM between incubation temperatures at embryonic stage 23. FPT data are represented as filled circles and MPT data are represented as open squares. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of ACTB) ± SEM.

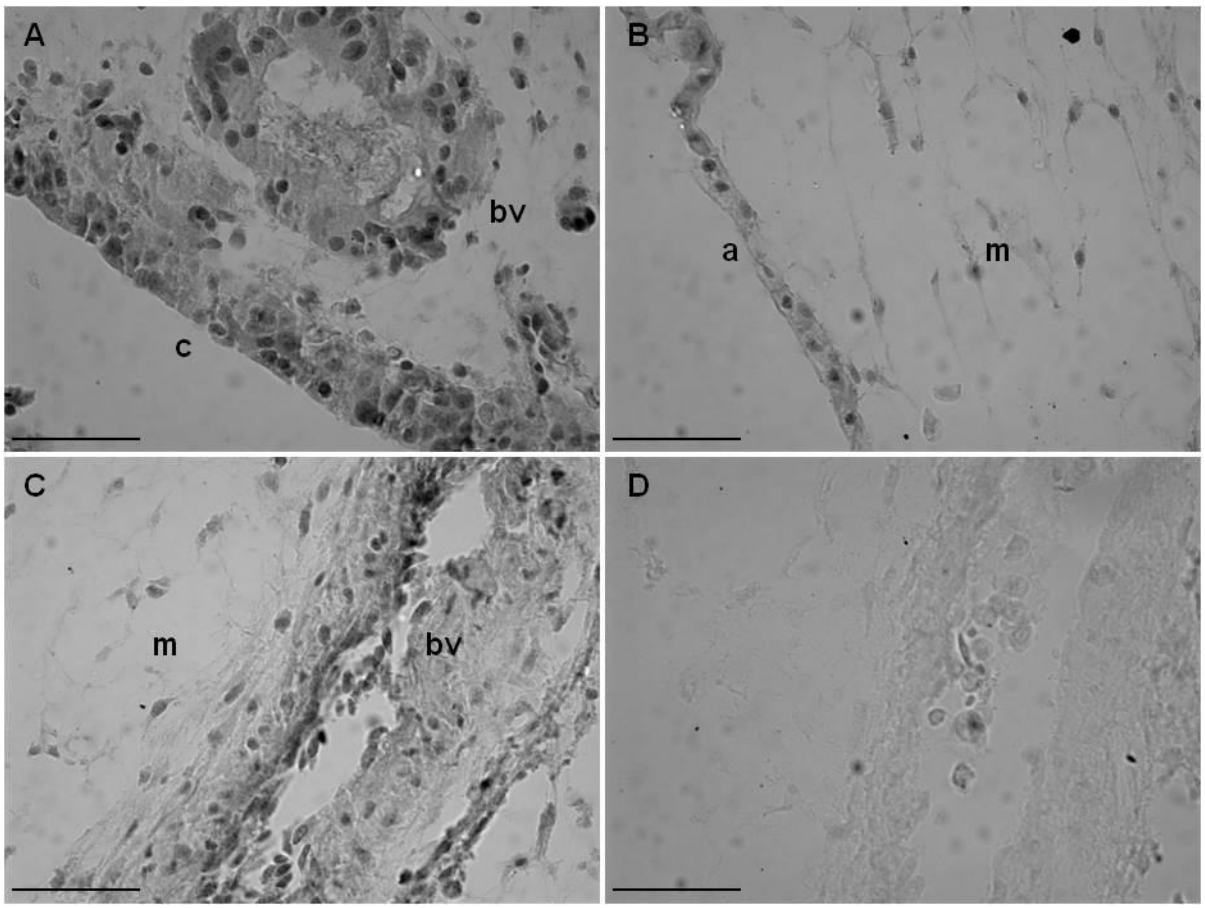


Figure 3-8. PR immunolocalization in the alligator CAM. (A) Positive nuclear staining of PR was localized to the chorionic epithelium (c) and epithelial cells of blood vessels (bv). (B) Nuclear staining was also observed in the allantoic epithelium and mesenchyme (m). (C) Positive CAM section corresponding to the (D) negative control incubated without primary PR antibody. Negative control CAM section did not show specific nuclear staining. Scale bar represents 50 microns.

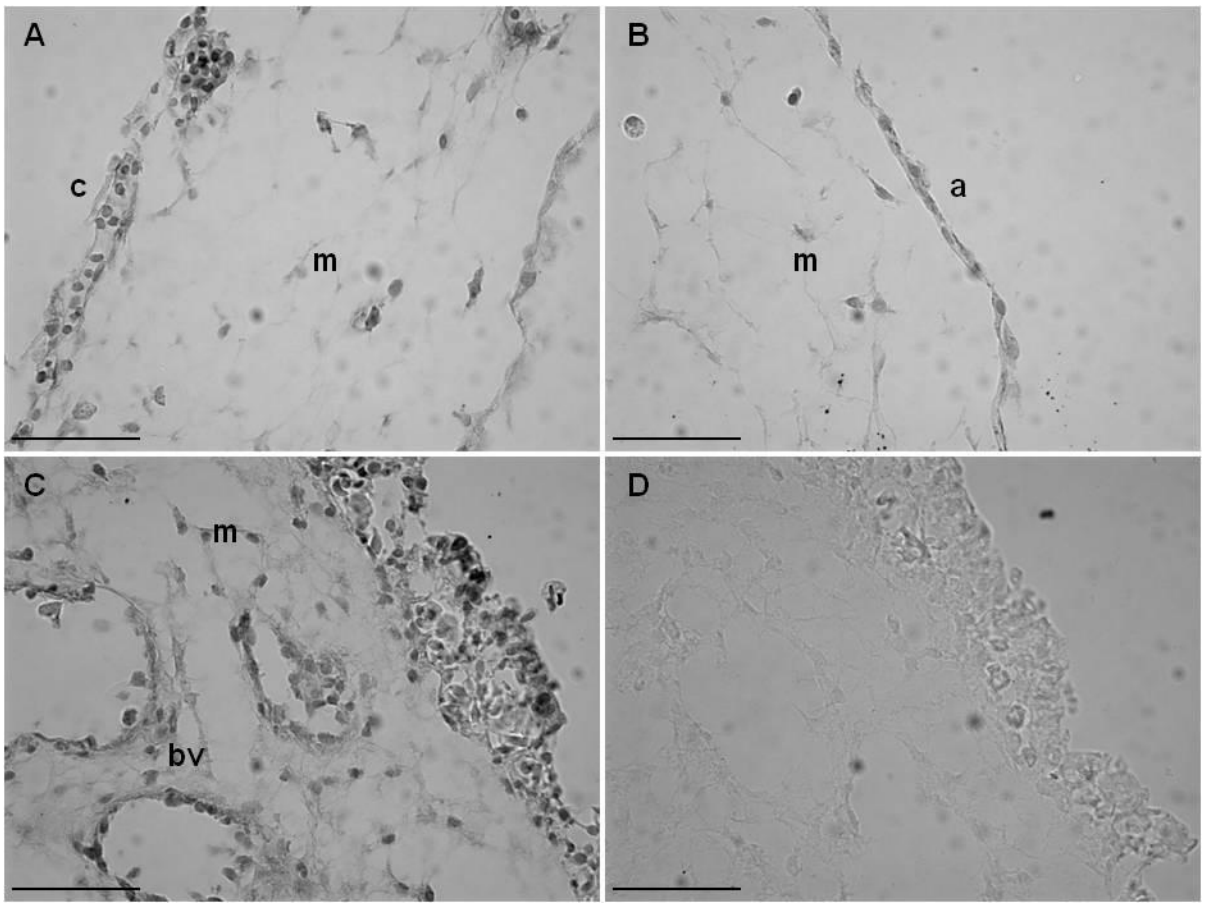


Figure 3-9. ESR1 immunolocalization in the alligator CAM. (A) Positive nuclear staining of ESR1 was localized to the chorionic epithelium (c) and mesenchyme (m). (B) Nuclear staining was also observed in the allantoic epithelium and (C) in the epithelial cells of blood vessels (bv). (D) Negative control of corresponding CAM section shown in (C) incubated without primary ESR1 antibody did not show specific nuclear staining. Scale bar represents 50 microns.

CHAPTER 4
THE OVIPAROUS CHORIOALLANTOIC MEMBRANE OF THE RED-BELLY SLIDER TURTLE (*PSEUDEMYS NELSONI*) HAS THE CAPABILITY TO SYNTHESIZE PROGESTERONE AND RESPOND TO STEROID HORMONE SIGNALING

The formation of a single extraembryonic membrane during development, the yolk sac, characterizes the ancestral condition of vertebrates [1, 56]. In addition to the yolk sac, the amniote (reptile, bird and mammal) ancestral condition is characterized by the formation of three additional extraembryonic membranes, the amnion, chorion and allantois [1]. These four extraembryonic membranes of the amniote egg gave rise to the placenta, which Mossman (1987) described as an apposition of extraembryonic membranes to uterine tissues “for the purpose of physiological exchange” [56].

Thus, it is not surprising that the amniote extraembryonic membranes of oviparous (egg-laying) species and placentae of viviparous (live-bearing) species perform similar functions to meet the gas, water, and nutrient demands of the embryo [56]. Of the four extraembryonic membranes, the chorion and allantois fuse early in embryonic development to form either the chorioallantoic membrane (CAM) in oviparous species or the chorioallantoic placenta in viviparous species [83]. Both the CAM and the chorioallantoic placenta are highly vascularized structures and perform respiratory functions by providing gas exchange between the developing embryo and its external environment [171, 172], i.e., the nest environment in oviparous species or the uterine environment in viviparous species.

We have previously stated ([36], Chapter 1), that this conservation of basic functions of amniote extraembryonic membranes indicates that little specialization of these membranes is required in the transition from oviparity to viviparity. This explains why the majority of viviparous squamates (lizards and snakes) exhibit a relatively simple

chorioallantoic placenta that appears to predominately function in gas exchange. The CAM and chorioallantoic placenta are organs of transport, not only of gases, but also of water and other inorganic ions essential for embryonic development, such as calcium, potassium and phosphorus ([173], as reviewed in [174]). In some viviparous species, the chorioallantoic placenta takes on additional roles in the transfer of nutrients, and in the most complex placental types, will transport all or the majority of nutrients the embryo requires for development (as reviewed in [174, 175]).

Still another transport function of the chorioallantoic placenta involves the transfer of steroid hormones between maternal and fetal environments [176]. Yet, the chorioallantoic placenta not only transports steroid hormones, but also synthesizes, metabolizes and responds to an array of hormones critical for embryonic development and survival [111, 153, 177, 178]. In eutherian mammals, progesterone (P4) synthesized by the placenta promotes uterine quiescence and the maintenance of pregnancy [179], timing of parturition [73], and fetal [74] and placental growth [75, 76]. Placental P4 synthesis is not an exclusive characteristic of eutherian mammals as the ability to synthesize P4 has been demonstrated in even the simplest chorioallantoic placenta of viviparous squamates [77], which is simply an apposition of the CAM to the uterine epithelium without any anatomical specializations in either structure [41]. Further, placental P4 synthesis is not an exclusive characteristic of the maternal contribution to the placenta and it is now evident that maternal and extraembryonic tissues alike contribute to steroidogenesis in mammalian [71, 78, 79] and lizard [21] placentae. Therefore, we hypothesized that CAMs of oviparous amniotes, sharing

conserved basic functions and evolutionary history, could also perform steroidogenesis and respond to steroid signaling [36, 116].

Albergotti et al. demonstrated that the CAM of one oviparous amniote, the chicken, shares the capability of chorioallantoic placentae to synthesize and respond to the signaling of P4 ([116], Chapter 2). The chicken CAM exhibited mRNA expression of steroidogenic enzymes involved in P4 biosynthesis, was capable of *in vitro* P4 synthesis, and exhibited mRNA and protein expression of the progesterone receptor (PR), which indicated that steroidogenic and steroid responsive extraembryonic membranes are not exclusive characteristics of viviparous amniotes ([116], Chapter 2). In addition, I have demonstrated that the CAM of another oviparous amniote, the American Alligator (*Alligator mississippiensis*), exhibits mRNA expression of steroidogenic enzymes and mRNA and protein expression of the PR and estrogen receptor α (ESR1) (Chapter 3). Our previous studies in the chicken and alligator suggest that the oviparous CAM of archosaurs shares the capability to synthesize steroid hormones at the molecular level and respond to steroid hormone signaling at the molecular and protein level. Collectively, this work supports our hypothesis that steroidogenesis and steroid hormone signaling of extraembryonic membranes could be an evolutionary conserved characteristic of amniotes.

Here, I moved beyond oviparous archosaurs and investigated the potential steroid activity in the CAM of the Red-belly Slider Turtle (*Pseudemys nelsoni*). The placement of turtles in the amniote phylogeny remains unresolved [180]. However, birds, crocodilians, turtles and the tuatara represent the main lineages of extant amniotes that reproduce strictly by oviparity [7, 180, 181]. The aim of our study was to investigate a

third branch of the oviparous amniote phylogeny in an attempt to uncover a potentially conserved trait of amniote extraembryonic membranes.

I examined the capability of the turtle CAM to perform steroid synthesis and respond to steroid hormone signaling. If the turtle CAM has similar steroidogenic properties as the chorioallantoic placenta, then it should synthesize key placental hormones, such as P4. To investigate steroid hormone synthesis, I examined the ability of the turtle CAM to perform *in vitro* P4 synthesis. Likewise, if the turtle CAM has similar steroid signaling properties as the chorioallantoic placenta, then it should have the capability to respond to signaling through steroid receptors. To investigate steroid hormone signaling, I examined turtle CAM expression of mRNA coding for PR, androgen receptor (AR), ESR1 and estrogen receptor β (ESR2), which are the key receptors responding to the signaling of progestins, androgens, and estrogens, respectively. In addition, to determine if steroid receptor mRNA is translated to protein, immunolocalization of the PR was examined.

Materials and Methods

Egg Collection

Annual reproduction and nesting of the Florida red-belly turtle roughly coincides with that of the American Alligator. It has been reported by others [182] and observed by myself that *P. nelsoni* routinely lay their eggs in the nest mounds of the American alligator. During the summers of 2007, 2008 and 2009, Florida red-belly turtle eggs were collected from alligator nests located within Lake Woodruff National Wildlife Refuge (Deland, Florida) and transported to the University of Florida for incubation. Within 48 hours of arrival, embryos from 1 to 2 eggs per clutch were used to determine the average embryonic stage of the clutch based on criteria described by Yntema [183].

Eggs from each clutch were incubated at a female producing temperature (FPT) of 30°C [184].

RNA Isolation and Reverse Transcription

In 2007 and 2009, CAMs were collected at embryonic stages 16, 19 and 22, and were dissected away from the embryo, yolk and eggshell, washed in 1X phosphate buffered saline (PBS) and stored in the RNA preservative, RNA*later* solution (Ambion) at 4°C overnight and stored at -20°C until RNA isolation. Total RNA was isolated from CAM with the SV Total RNA Isolation System (Promega), and reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad). Concentrations and quality of RNA samples were evaluated by measuring optical density with a NanoDrop ND-1000 (Thermo Scientific) and by formaldehyde gel electrophoresis. Total RNA was treated with ribonuclease-free deoxyribonuclease I (DNase I; Qiagen) to remove any genomic DNA contamination. One µg of total RNA was reverse transcribed in 20 µL reaction and complementary DNA (cDNA) was stored at -20°C until RT-qPCR analysis.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR analysis was performed on CAM samples from different clutches at embryonic stages 16 (n=18), 19 (n=12) and 22 (n=19). Complementary DNA was analyzed in triplicate by RT-qPCR amplification using an iCycler MyIQ Single Color Real-Time PCR Detection System (Bio-Rad). Each 15-µL DNA amplification reaction contained 10 mM Tris-HCl (pH 7.84), 50 mM KCl, 3 mM $MgCl_2$, 200 µM dNTPs, 0.5% Tween-20, 0.8% glycerol, 2% DMSO, 200-fold dilution of SYBR Green (Invitrogen), 0.01 µM Fluorescein Calibration Dye (Bio-Rad), 0.2 µM of each primer, 0.01 U/µL AmpliTag Gold DNA polymerase (Applied Biosystems) and 25-fold dilution of cDNA. RT-qPCR amplification conditions included an enzyme activation step of 95°C (8 min) followed by

40 cycles (internal control genes) or 50 cycles (target genes) of 95°C (15 sec) and a primer specific combined annealing/extension temperature (1 min). The specificity of amplification was confirmed by melt-curve analysis.

Triplicate data for each gene were averaged and mRNA expression levels of the steroid receptors (PR, AR, ESR1, ESR2) were determined by the absolute quantification method [87]. In brief, copy numbers were calculated from the cycle threshold (Ct) value by the linear regression of an absolute standard curve. Absolute standard curves for each target gene were generated from a plasmid containing the amplicon of interest at known concentrations. Controls lacking cDNA template were included on every RT-qPCR plate to determine the specificity of target cDNA. Additionally, to confirm that target cDNA was not contaminated by genomic DNA, RT-qPCR was performed with protein phosphatase 1 gamma (PP1) primers on the RNA isolated from every sample. All sample means were normalized using PP1 expression. Data are reported as relative expression and represent mean normalized mRNA transcript percentage in (% of PP1).

Cloning and Sequencing of Plasmids

RT-qPCR of pooled cDNA was used to generate a PCR product for each primer set. Amplified PCR products were separated on a 2% agarose gel and visualized by ethidium bromide on a Gel Doc EQ with Quantity One 4.6 software (Bio Rad). RT-qPCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega) and purified samples were confirmed by electrophoresis on a 2% agarose gel. PCR products were cloned into a pGEM®-T Vector System (Promega). Plasmid DNA was purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega) and sequenced on an ABI PRISM® 3130 Genetic Analyzer (Applied Biosystems) using a

BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). The specificity of cloned DNA was confirmed using BLAST against sequences available in Genbank. Clone DNA concentration was quantified by NanoDrop™ ND-1000, converted to copies/µL, and serially diluted in a solution containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, and 5 µg/mL of tRNA.

RT–qPCR Primers

All primers were designed using Primer3 software [36] and were synthesized by Eurofins MWG Operon. With the exception of PP1, all primers were designed to amplify mRNA-specific fragments from *P. nelsoni* coding sequences (NCBI). For PP1, primers were designed from a partial mRNA sequence for *Trachemys scripta* (DQ848991.1), cloned and sequenced as described above. Blast results indicated a (109/111) 98% identity in PP1 sequences between *T. scripta* and *P. nelsoni*. The two single nucleotide differences between the species were not found in either the forward or reverse primer region, allowing the use of this primer set for *P. nelsoni*. All primer pairs were combined and diluted to a final concentration of 10 µM. Primer sequences, annealing/extension temperatures and GenBank accession numbers are reported in Table 4-1.

***In Vitro* Explant Culture**

In 2008, CAMs were collected at embryonic stages 23 to 24. Sections of CAM were cut to approximately 0.1g wet weight (mean= 0.108g ± 0.001SEM). CAM sections were incubated at 30°C on an orbital shaker in L-15 culture media (Invitrogen) either with (n=18) or without (n=21) cholesterol and cAMP as precursor. Precursor solutions and concentrations are based on King et al. 2004 [91]. For cholesterol, 22(R)-Hydroxycholesterol (Sigma) was dissolved in 95% ethanol (Fisher) to a final concentration of 10µg/mL and combined with 1 mM Dibutyryl cAMP (Sigma). After 8

hours of incubation, concentration of progesterone in the culture media was quantified by solid phase radioimmunoassay [92]. To determine background and cross-reactivity of the P4 assay, controls consisting of only cholesterol and cAMP (absence of CAM) were incubated for 8 hours. In the absence of CAM, all samples were below the limit of detection of the assay.

Histology, Immunohistochemistry and Microscopy

In 2009, dissected CAM was fixed in neutral buffered formalin, washed in 1X PBS and stored in 75% ethanol. CAMs from stage 16 (n=3), stage 19 (n=3) and stage 22 (n=3) were dehydrated, paraffin embedded, and sectioned at 5 microns. Standard hematoxylin and eosin staining was performed with stains from Surgipath. Immunohistochemistry was performed using the Elite ABC Mouse IgG Kit (Vector Laboratories). Tissue sections were deparaffinized in xylene, transferred to 100% ethanol, treated with 3% hydrogen peroxide in methanol (10 min) and rehydrated through graded concentrations of ethanol to dionized water (dH_20). Antigen retrieval was performed by heating slides to 95 to 97°C(25 min) in Trilogy solution (Cell Marque), rinsed in dH_20, and placed in 0.1 M Tris buffered saline containing 0.2% Tween 20 (TBS-T) for 5 minutes. Sections were treated with the Avidin Biotin blocking kit (Vector Laboratories) and then blocked in normal serum (20 min). Sections were incubated with a 1:100 dilution of mouse monoclonal anti- progesterone receptor antibody (Ab-8) (Thermo Scientific) overnight at 4˚C. Sections were incubated with secondary antibody (30 min) and ABC reagent (5 min). Slides were washed in TBS-T between all incubation steps. Sections were treated with 3, 39-diaminobenzidine (5 min) (Vector Laboratories) and washed in water (5 min). A negative control section receiving concentration matched IgG controls were included on every slide. Slides were dehydrated, cleared

and mounted with Cytoseal™ XYL Mounting Medium (Richard-Allan Scientific®). Sections were imaged using an Olympus BX60 microscope under differential inference contrast (DIC) and Zeiss AxioCAM MR3 camera with Zeiss AxioVision LE 4.8.2 software.

Statistical Analysis

All statistical analyses were performed in the R statistical programming environment version 2.13.1 [95]. For gene expression analyses, the total numbers of mRNA transcripts for 1 internal control and 4 target genes from the CAM were determined by RT-qPCR. To quantify relative expression of target genes, each sample was normalized by PP1 expression. To analyze each target gene, I used linear mixed effects models (LMMs) to compare relative mRNA expression over the three stage experiment. For each analysis, embryonic stage was treated as a fixed effect, and RT-qPCR plate, maternal clutch and collection year were treated as random effects. Collection year did not significantly reduce the deviance in the data explained by the model and was excluded from the model for all genes. Likewise, maternal clutch did not significantly reduce the deviance in the data explained by the model and was excluded from the model for all genes except AR. Model assumptions were evaluated visually via examination of residuals and QQ plots and log transformations were performed on all target genes. The assumption of homogeneity of variances was violated for two genes (PR and ESR1) and so I weighted the variance for each stage according to a power law function that was estimated from the data as part of the model fitting procedure [96].

For *in vitro* tissue culture, an unpaired, two-sided Student's t-test was used to compare the mean concentration of P4 in the culture media from CAMs incubated with L15 media only (absence of precursor) to CAMs incubated with L-15 media plus

cholesterol and cAMP (precursor). The assumption of normality was evaluated with a two-sided F-test. A Student's t-test assuming unequal variances was also used to compare the concentration of P4 in the culture media from CAMs with L15 media only (absence of precursor) to a hypothetical mean of zero.

Results

The Turtle CAM is Capable of *In Vitro* Progesterone Synthesis

The effect of steroid precursor addition on the concentration of P4 in culture media was analyzed. There was a significant increase in the concentration of P4 in the culture media following the addition of cholesterol precursor plus cAMP ($t(37)=2.77$, $p=0.008$, mean=1362.18 pg/mL/g ± 126.32 SEM) (Figure 4-1). Further, a significant concentration of P4 in the culture media in the absence of precursor, but in the presence of CAM was found ($t(20)= 11.57$, $p<0.0001$, mean=955.2 pg/mL/g ± 82.57 SEM) (Figure 4-1).

The Turtle CAM is Capable of Responding to Steroid Hormone Signaling

The relative levels of steroid receptor mRNA in the CAM exhibited the pattern ESR1 > ESR2 > AR > PR (Figure 4-2). At all three embryonic stages, ESR1 was the most highly expressed steroid receptor and PR was the lowest. The relative levels of steroid receptor mRNAs were separated by an order of magnitude or greater.

The effect of embryonic stage on the level of mRNA expression was analyzed in the turtle CAM. Overall, there was significant mRNA expression of PR ($F_{1,45}$ = 3932.05, $p <0.0001$), AR ($F_{1,33}$ = 1235.5, $p <0.0001$), ESR1 ($F_{1,45}$ = 742.9, $p <0.0001$) and ESR2 ($F_{1,45}$ = 669.5, $p <0.0001$). However, the level of expression did not change significantly among embryonic stages for ESR1 ($_{2,45}$ = 2.52, $p =0.0916$) and ESR2 ($F_{2,45}$ = 2.72, $p =0.0764$) (Figure 4-2). In contrast, PR expression increased 2.3 fold ($F_{2,45}$=3.67,

p=0.0335) and AR expression increased 7.0 fold ($F_{2,33}$=29.36, p<0.0001) between embryonic stages 16 and 22 (Figure 4-2). To determine if PR mRNA was translated to protein, immunolocalization of the nuclear PR was performed. Nuclear PR staining was evident throughout the chorionic and allantoic epithelia, mesenchyme and epithelial cells of mesenchymal blood vessels (Figure 4-3).

Discussion

Oviparity is the ancestral reproductive mode of amniotes [56, 172, 185]. Within this group of vertebrates; birds, crocodilians, turtles and the tuatara represent the major lineages of extant amniotes that reproduce strictly by oviparity [7, 180, 181]. In contrast, both squamate and mammalian lineages are represented by two reproductive modes, with some species reproducing by oviparity and others reproducing by viviparity [181] (Figure 4-4). We previously hypothesized that endocrine extraembryonic membranes are not an exclusive trait of viviparous amniotes, but rather are a conserved characteristic of amniotes independent of reproductive mode [36, 116]. To that end, steroid synthesis and/or steroid signaling potential has now been demonstrated in the CAMs of two oviparous species, the chicken and alligator ([116], Chapter 2, Chapter 3). Our current study examined steroid hormone synthesis and signaling in a third branch of the oviparous amniote phylogeny. Here, I demonstrate that the turtle CAM can perform steroid synthesis and has steroid hormone signaling capabilities (Figure 4-4), lending further support for our hypothesis that endocrine activity of extraembryonic membranes is a conserved trait of amniota.

Albergottti et al. were the first to demonstrate that the oviparous CAM of chicken has the capability to perform *in vitro* P4 synthesis ([116], Chapter 2). Our current study shows that *in vitro* P4 synthesis can also be induced in the CAM of another oviparous

amniote, the Florida red-belly slider turtle. To be confident that the P4 quantified in the culture media was synthesized by the turtle CAM, I removed CAM tissue from the egg and tested for synthesis directly in the presence of a steroid hormone precursor. I hypothesized that if the CAM was steroidogenic, then addition of the steroid hormone precursor to the culture media would stimulate increased P4 production. Our results showed a significant increase in the concentration of P4 in the culture media following the addition of precursor (Figure 4-1), confirming that P4 synthesis can be induced in the turtle CAM. In addition, I found a significant concentration of P4 in the culture media in the absence of precursor suggesting that the turtle CAM exhibits endogenous P4 synthesis. However, the CAM is a highly vascularized tissue and it is possible that the P4 detected in the culture media in the absence of precursor could be the result of P4 synthesized by or mobilized from a source other than the CAM, which simply leached from the CAM to the culture media at the time of dissection. While, the data were not able to tease apart the question of endogenous P4 synthesis at this time, the power of this experimental approach is its ability to definitively show that the turtle CAM has the ability to synthesize P4 in the presence of a steroid hormone precursor.

The concentrations of P4 in the culture media reported in the turtle CAM assay are substantially higher than those previously reported in the chicken ([116], Chapter 2). In the presence of precursor, the average concentration of P4 in the culture media after 8 hours of incubation was 101.12 pg/mL/g ± 10.22 in the chicken as compared to 1362.18 pg/mL/g ± 126.32 in the turtle and in the absence of precursor was 43.65 pg/mL/g ± 7.14 in the chicken and 955.2 pg/mL/g ± 82.57 in the turtle. It seems highly unlikely that the extreme differences in concentration of P4 between these two studies

could be explained by laboratory procedures. While a different shipment of the P4 antibody used in the radioimmunoassay between these studies could introduce some variation in the results, the Guillette laboratory has a long history with this technique and different shipments of antibodies produce consistent results with values between shipments being less than two times different. Our results indicate that the concentration of P4 in the culture media was 13 times higher in the presence of precursor and 22 times higher in the absence of precursor in the turtle as compared to the chicken. This suggests that there are considerable differences in the CAMs ability to synthesize P4 and perhaps in the level of endogenous P4 production between these two species, at least late in embryonic development when these experiments were performed. Species differences in CAM P4 synthesis might be influenced by differences in circulating levels of maternally deposited yolk hormones or those synthesized by the embryo, as well as the levels the steroidogenic enzymes and steroid receptors expressed in the CAM.

Steroid hormone biosynthesis begins with cholesterol and requires the action of specific steroidogenic enzymes to convert one hormone to another. In the biosynthesis of P4, cholesterol is converted to pregnenolone (P5) by the action of cytochrome P450 11A1 (CYP11A1). P5 can then be converted to P4 by the action of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (HSD3B1) [97] (Figure 4-5). Thus, the turtle CAMs demonstrated ability to synthesize P4 from cholesterol indicates that the steroidogenic enzymes, CYP11A1 and HSD3B1, are present and functional in this tissue. CYP11A1 and HSD3B1 mRNAs have previously been demonstrated in the chick CAM and HSD3B1 in the alligator CAM ([116], Chapter 2,

Chapter 3). Further, the chick CAM also demonstrated *in vitro* synthesis of P4 from cholesterol ([116], Chapter 2). Therefore, the CAMs of three oviparous amniotes have now been shown to express steroidogenic enzymes either at the molecular level alone (alligator), the molecular and functional level (chicken) or the functional level alone (turtle); thus, supporting our hypothesis that a conserved characteristic of the oviparous CAM includes the capability to perform steroidogenesis (Figure 4-4).

The importance of placental P4 in the maintenance of pregnancy is well established in eutherian mammals [179]. However, oviparous amniotes, such as birds, crocodilians and turtles lay their eggs at early embryonic stages prior to the development of extraembryonic membranes [53, 86, 129, 183] indicating that this would not be a logical function of CAM P4 in these species. Yet, if we consider additional roles of placental P4, we find potential functions of P4 in the oviparous CAM. First, P4 promotes protein synthesis and general growth [186], both of the placenta [74-76] and of the fetus [74]. In addition, P4 stimulates the proliferation of blood vessels [107] and plays a role in fetoplacental vascularization [109, 110]. The CAM, like the chorioallantoic placenta, undergoes dramatic growth during development and functions as a support structure for the growing embryo. Thus, one could hypothesize that P4 synthesized by the CAM could play a role in promoting CAM and embryonic growth. Moreover, both the CAM and chorioallantoic placenta are highly vascularized organs; therefore, it seems reasonable to suggest that P4 could have a role in CAM blood vessel proliferation and vascularization. For example, vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) are important regulators of placental [187] and CAM angiogenesis [188, 189]. P4 has been shown to induce the expression of VEGFs [190] and FGFs

[191]; therefore, it would be interesting to know how P4 affects VEGF and FGF expression and vascularization in the CAM. I suggest that future work should investigate these processes as first steps in determining potential functions of P4 in the oviparous CAM.

Progestins, estrogens and androgens exert their actions on target cells primarily through binding and activation of the PR, estrogen receptors (ERs) and AR, respectively. As members of the steroid hormone superfamily, these receptors act as ligand-dependent transcription factors to regulate gene expression [84, 192]. Steroid hormone receptor mRNA and protein expression was demonstrated in the oviparous CAMs of chicken and alligator ([116], Chapter 2, Chapter 3). Here, for the first time, I show the presence of PR, AR, ESR1 and ESR2 mRNA and protein expression of PR in the CAM of a turtle (Figure 4-2, Figure 4-3). The presence of steroid receptor mRNA and protein in the turtle CAM lends additional support for our hypothesis that the capability to respond to steroid hormone signaling is a conserved characteristic of the oviparous CAM. In the turtle CAM, the relative levels of mRNA were separated by an order of magnitude or more and exhibited the pattern ESR1 > ESR2 > AR > PR. The levels of steroid receptor expression found here could reflect the relative importance of the turtle CAM to be able to respond to estrogens, androgens and progestins, respectively. Yet, it is well recognized that mRNA expression does not always result in protein expression. Further, transcript levels do not necessarily reflect translation levels. Therefore, future studies are needed to examine the translation of the steroid receptor mRNAs into active proteins before we can establish function of PR, AR and the ER's or true signal reception of AR and the ER's in the turtle CAM.

Placental [126, 127, 193] and CAM ([116], Chapter 2, Chapter 3) steroidogenic enzyme and steroid receptor mRNAs have been observed to change during development. Therefore, I investigated steroid receptor mRNA expression in the turtle CAM at three embryonic stages corresponding to before (stage 16), during (stage 19) and after (stage 22) the thermo-sensitive period (TSP) of sex determination. Embryonic stages surrounding the TSP were chosen with the hypothesis that this developmental period would correspond to increased steroid signaling activity in the embryo and CAM, thereby increasing the chances of detecting and quantifying differences in CAM steroid receptor mRNAs. Overall, I found that PR and AR exhibited changes among the embryonic stages suggesting that steroid receptor mRNA expression is affected by developmental stage in these two genes.

In the turtle CAM, both PR and AR showed increased expression at stage 22 relative to stage 16. This pattern is similar to the one observed in the chick CAM, where PR exhibited very low expression early in development, but increased substantially between day 8 and 18 of development ([116], Chapter 2). However, the pattern of expression in the turtle CAM contrasts with that of the alligator, which showed a decrease in PR and AR mRNAs between embryonic stage 19 and 25 in CAM's incubated at the male producing temperature and in AR at the female producing temperature (Chapter 3). Currently, there is very little developmental data on placental steroid receptor expression through gestation. However, in the junctional zone of the rat chorioallantoic placenta, PR binding sites increased between days 14 and 20 of pregnancy [178]. Thus, it appears that the chick and turtle CAM, by demonstrating increased PR mRNA expression during the developmental period examined, could

resemble the pattern of PR in the rat placenta, but without protein analyses in the CAMs these studies are not directly comparable. Unfortunately, developmental expression patterns of AR in the placenta are not available for comparison.

It is well recognized that vertebrate yolk contains maternally derived steroid hormones deposited into the egg at oviposition [99, 160, 163]. However, how yolk steroid hormones are mobilized to and what functions they provide for the developing embryo is less clear. It is becoming increasingly evident; however, that yolk hormones are mobilized to and metabolized by the embryo [167] and extraembryonic membranes [162, 170]. Thus, we have hypothesized that the CAM can respond to and biotransform circulating yolk steroid hormones in addition to those synthesized by the embryo and CAM [36]. Concentrations of yolk steroids, such as estradiol 17β (E2), testosterone (T) and P4, are typically highest at oviposition and subsequently decline during embryonic development [155, 161, 163]. Thus, yolk steroids might be important early sources of hormones prior to steroidogenic capability coming on line in the embryo or CAM, an idea that is supported by the presence of steroid receptor mRNAs in the embryo prior to it's ability to perform steroid synthesis [194]. Yolk steroids could be biotransformed by the CAM to synthesize different hormone products and/or for the degradation of biologically active hormones to less active forms, thus acting to buffer embryonic exposure to potentially harmful levels of steroid signals at inopportune times in development [36].

Moreover, we previously hypothesized that the oviparous yolk sac membrane (YSM), in addition to the CAM, would have the ability to respond to and biotransform maternally deposited yolk steroids [36]. The bird YSM functions in the transport and

conversion of maternally deposited lipids in the yolk to the embryo [195, 196]. Therefore, I build on our previous hypothesis, which suggested that in mobilizing yolk lipids and steroids, the YSM could deliver these substances to the CAM, thereby providing substrates for steroidogenesis in this tissue [36], and further hypothesize that the YSM also has the capability itself to metabolize yolk steroids and perform steroidogenesis (Figure 4-6). In support of this hypothesis, I have recently observed that the chick YSM exhibits mRNA expression of steroidogenic enzymes and steroid receptors and protein expression of PR and ESR1 (Albergotti, unpublished data) (Figure 4-4).

Collectively, our hypothesis suggests that the oviparous amniote has an integrated system of steroid synthesis and metabolism in which the embryo and extraembryonic membranes function together to regulate the steroid milieu of the developing embryo (Figure 4-6). Moreover, we have suggested that this system has been co-opted and modified to serve a viviparous mode of reproduction, but likely did not evolve in response to viviparity [36]. Rather, steroidogenesis and steroid signaling in extraembryonic membranes is likely older than even amniotes, as the ancestral condition of vertebrates is characterized by the formation of a single extraembryonic membrane, the YSM [1, 56] and the yolk of fishes and amphibians contains signaling molecules, such as steroid, thyroid and retinoid hormones that are mobilized to the developing embryo ([197-203], Fujikura and Suzuki, 1991 as cited by [204]).

Collectively, our study indicates that the turtle CAM has the capability to perform *in vitro* P4 synthesis, respond to androgens and estrogens at the molecular level, and respond to progestins at the molecular and protein level. Future studies are needed to

better understand steroidogenesis and steroid signaling in the oviparous CAM as well as the YSM. I suggest that protein and functional assays are of the upmost importance in future investigations. While immunolocalization of the PR has been demonstrated in three oviparous CAMs to date, demonstration of protein expression of key steroidogenic enzymes are lacking. Likewise, I suspect that the CAM has the capability to synthesize not only P4, but also estrogens, androgens and glucocorticoids and future work should investigate extraembryonic membrane synthesis of these and other peptide hormones associated with the chorioallantoic placenta.

Table 4-1. Turtle PCR primers used for RT-Quantitative real-time PCR

Gene	Direction	Sequence (5'-3')	Annealing (C)	GenBank accession number
PP1	Sense	TCCTGCTGGCCTACAAGATT	63.7	DQ848991.1
	Antisense	CGCCTCTTGCACTCATCATA		
PR	Sense	CCAGCATGTCGATTGAGAAA	60	AB301062.1
	Antisense	GCTGCTGGAGTGCAACAATA		
AR	Sense	ATGTCCTGGAAGCCATTGAG	68	AB301061.1
	Antisense	CTCTCCCCAAGCTCATTCAG		
ESR1	Sense	ATCATTTGGGTCCAGCAGTC	60.5	AB301060.1
	Antisense	TAAGAATACCACGGGGCTTG		
ESR2	Sense	ACGCTTCGAGGTCAAAGAAA	60	AB548299.1
	Antisense	GCCATATTCCGCGTATCATC		

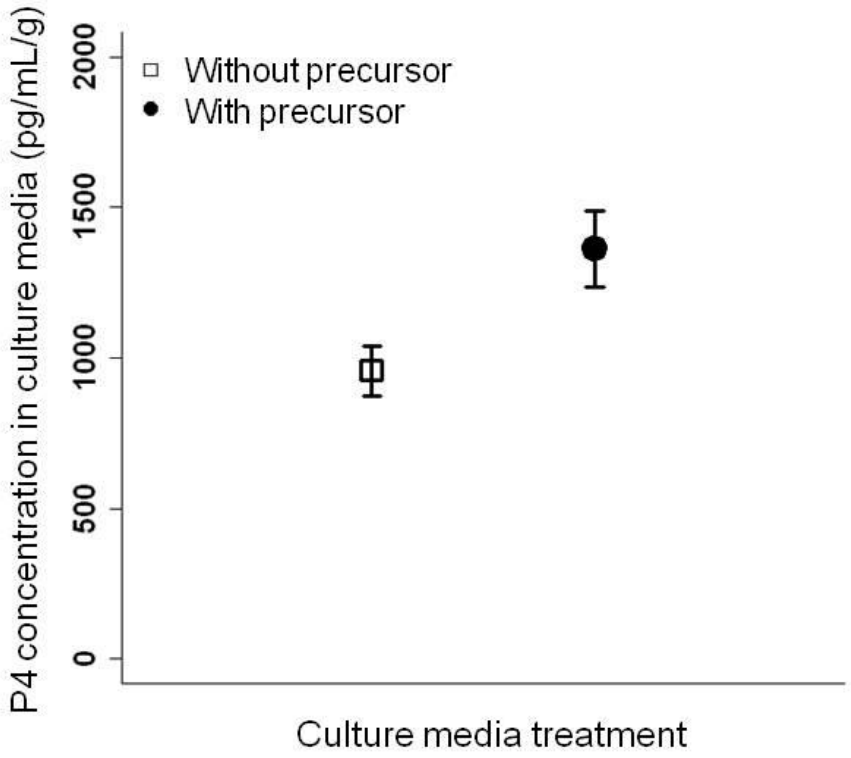


Figure 4-1. Progesterone synthesis in the turtle CAM. CAM sections were incubated in culture media for 8 hours either without (open squares) or with (filled circles) cholesterol (plus cAMP) as a precursor. Concentration of P4 in the culture media is represented as pg/mL of P4 per g of CAM tissue (pg/mL/g). To determine background and cross-reactivity of the P4 assay, controls consisting of only cholesterol and cAMP (absence of CAM) were incubated for 8 hours. In the absence of CAM, all samples were below the limit of detection of the assay.

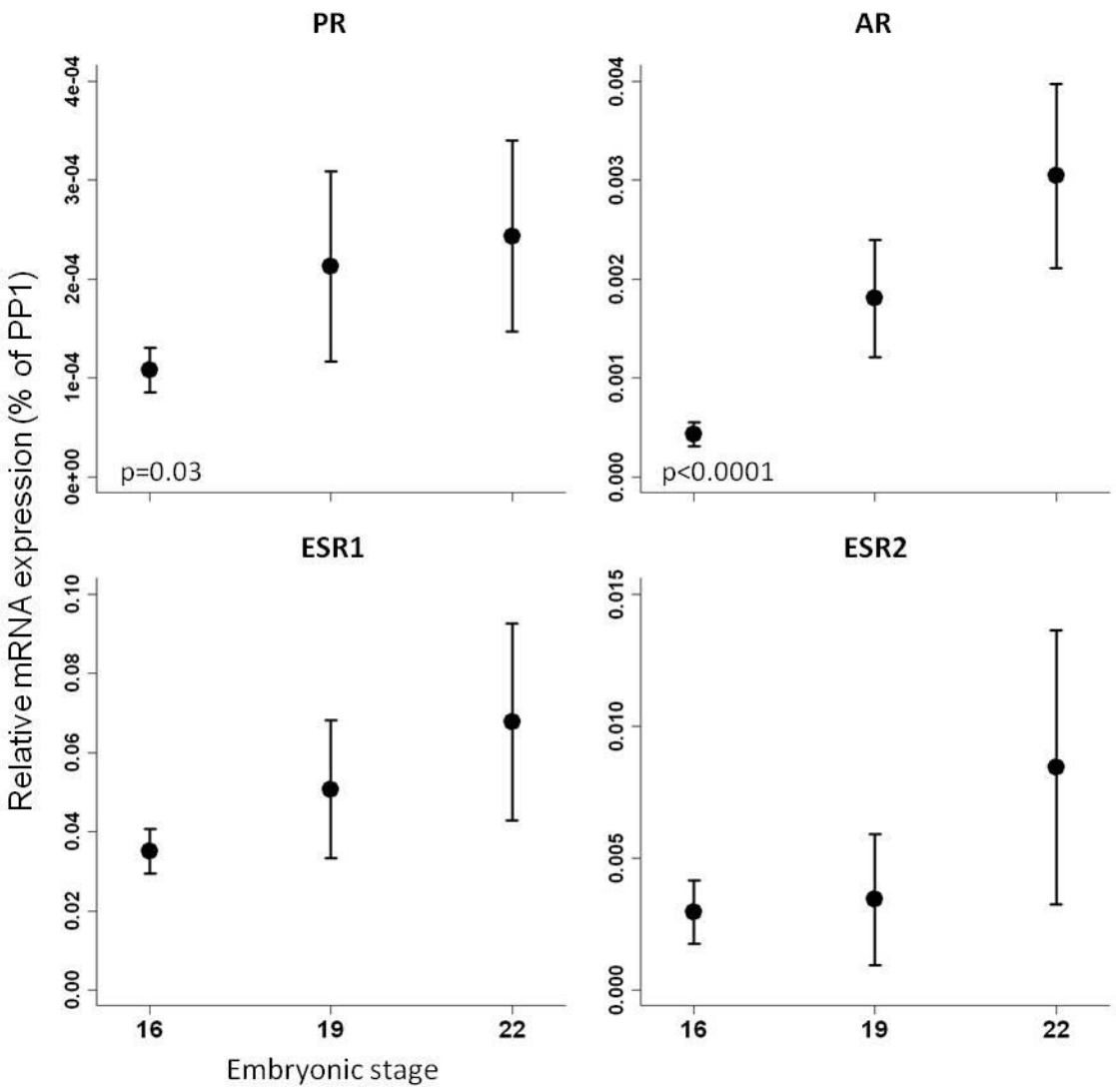


Figure 4-2. Relative mRNA expression of steroid receptors in the turtle CAM. RT-qPCR analysis of mRNA coding for, PR, AR, ESR1, and ESR2 at embryonic stages 16, 19, and 22. Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of PP1) ± SEM.

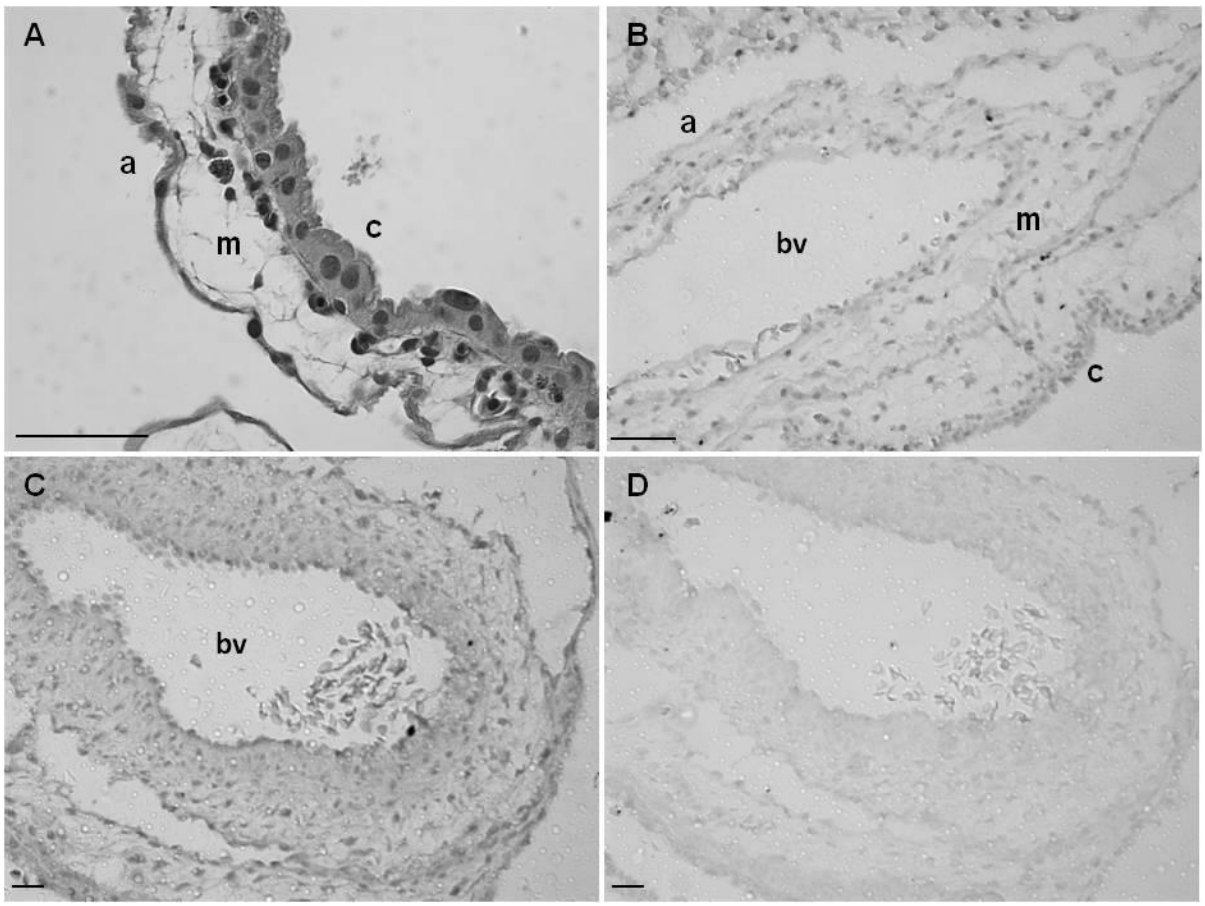


Figure 4-3. Histology and immunolocalization of PR in turtle CAM. (A) H&E staining in the turtle CAM showing the allantoic epithelium (a), mesenchyme (m) and chorionic epithelium (c). (B) Positive nuclear staining of PR was localized to both epithelial layers, the mesenchyme and to the epithelial cells of blood vessels (bv). (C) Positive CAM section corresponding to the (D) negative control receiving concentration matched IgG. Scale bar represents 50 microns.

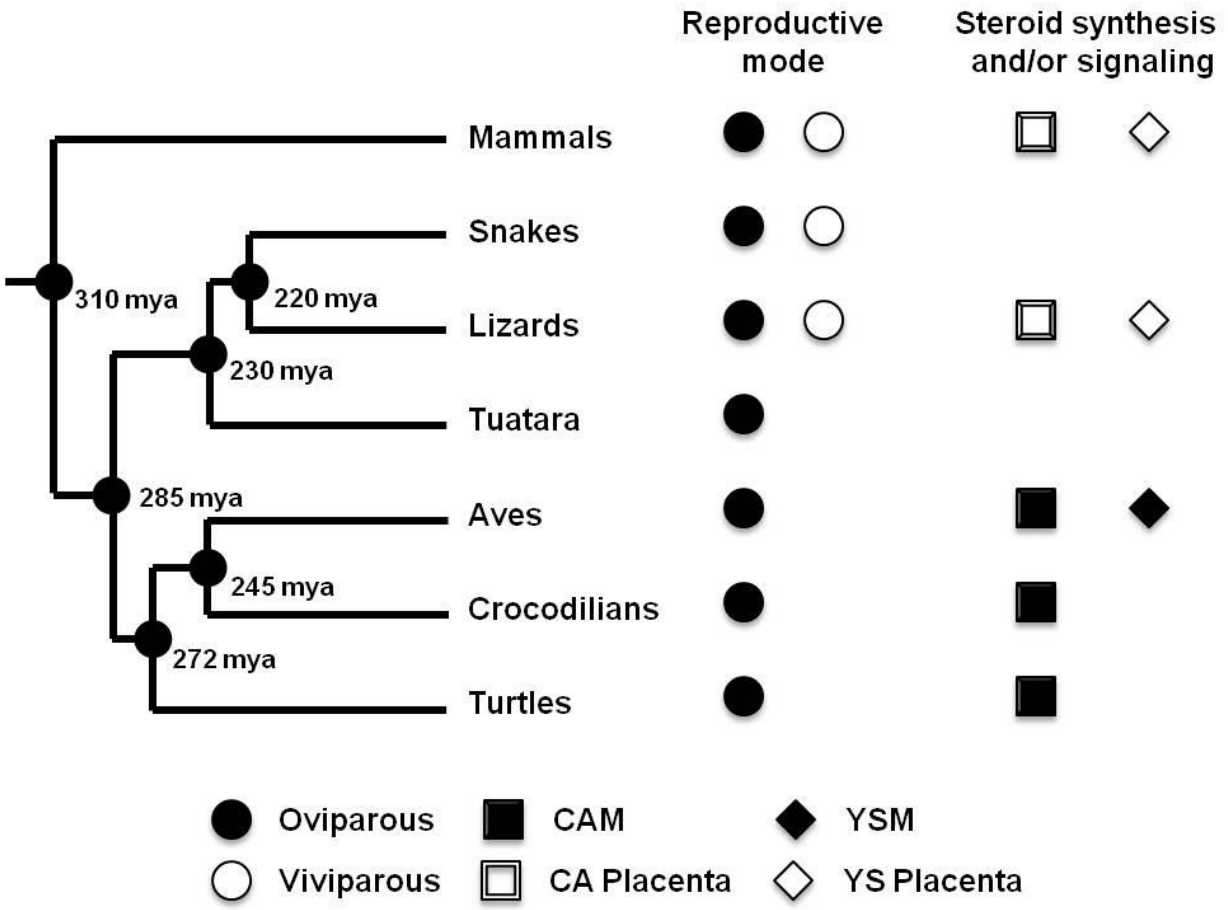


Figure 4-4. Amniote reproductive modes and evidence of steroid hormone synthesis and/or steroid signaling in extraembryonic membranes and placentae. Birds, crocodilians, turtles and the tuatara represent the major lineages of extant amniotes reproducing only by oviparity (filled circles); whereas, lizards, snakes and mammalian lineages are represented by two reproductive modes, with some reproducing by oviparity and others reproducing by viviparity (open circles). Previous studies have demonstrated that the chorioallantoic placenta (open squares) and the yolk sac placenta (open diamonds) have the ability to perform steroid synthesis and/or respond to steroid hormone signaling in the viviparous lineages noted. There have been no investigations of such in snakes. We have previously demonstrated that the oviparous CAM (filled squares) of chicken and alligator has the capability to perform steroidogenesis and/or respond to steroid hormone signaling. This study demonstrates that this capability extends to a turtle. Further, we have observed that this capability is also evident in the chick YSM (filled diamonds) (Albergotti, unpublished data). Taken together, these findings suggest that endocrine extraembryonic membranes could be conserved characteristics of amniota. Phylogeny adapted from Ezaz T, Stiglec R, Veyrunes F, Marshall Graves JA. Relationships between vertebrate ZW and XY sex chromosome systems. Curr Biol 2006; 16:R736-R743 [205].

Figure 4-5. A simplified version of progesterone biosynthesis. Filled boxes highlight steroidogenic enzymes required for the conversion of cholesterol to progesterone (P4). Cholesterol is converted to pregnenolone (P5) by the action of CYP11A1. P5 can then be converted to P4 by the action of HSD3B1.

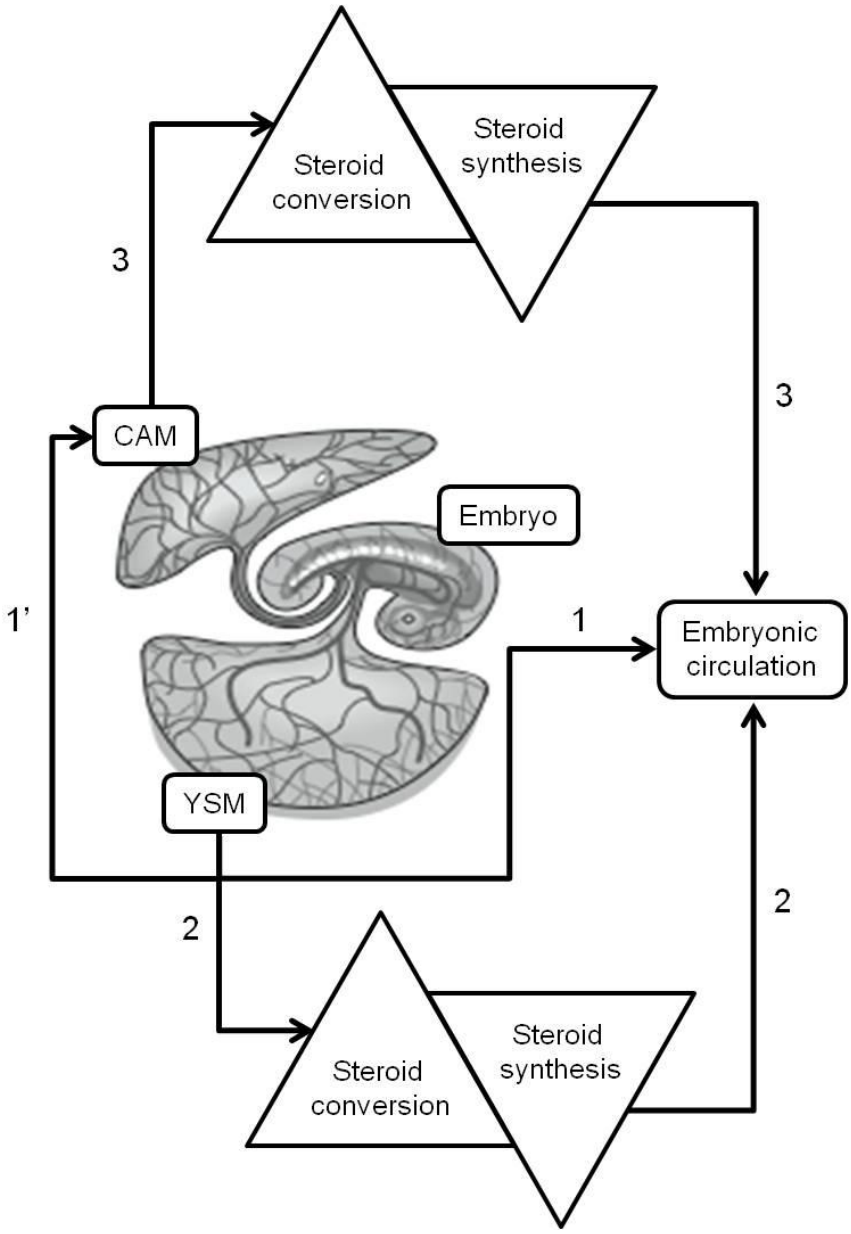


Figure 4-6. Model for the evolution of endocrine function in the amniote yolk sac (YSM) and chorioallantoic membrane (CAM). This model proposes that initially the YSM mobilized yolk lipids and steroids to embryonic circulation (pathway 1). With the evolution of amniotes, the YSM also mobilized yolk lipids and steroids to the CAM (pathway 1'). Subsequently, the YSM (pathway 2) and CAM (pathway 3) acquired the ability to convert yolk steroids to 'protect' the developing embryo and/or provide 'developmental signaling' as well as the ability to synthesize steroid hormones *de novo* prior to the evolution of viviparity. Figure adapted from Albergotti LC, Guillette LJ, Jr. Viviparity in reptiles: evolution and endocrine physiology. In: Norris DO, Lopez KH (eds.), Hormones and reproduction in vertebrates, vol. 3: Elsevier; 2011: 247-275. [36].

CHAPTER 5
ESTROGENIC EXPOSURE AFFECTS CHORIOALLANTOIC MEMBRANE GENE EXPRESSION AND TIMING OF HATCH IN THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*)

The highly vascularized chorioallantoic membrane (CAM) is formed by the fusion of two extraembryonic membranes, the chorion and allantois [55]. In viviparous, i.e. live-bearing amniotes (mammals, reptiles and birds), the CAM apposes the maternal uterus and together these tissues form the chorioallantoic placenta. The chorioallantoic placenta is a transient organ that serves as in the maternal-fetal exchange of gases, metabolic wastes, nutrients and chemical messengers [56]. In the absence of contact with maternal circulation, the CAM of the self-contained oviparous egg serves as the major site of respiration, as a repository for metabolic wastes [55], and in the transfer of essential electrolytes [173]. Following parturition, the placenta is no longer needed and is discarded at birth. Similarly, the CAM is discarded at hatching and much of the CAM tissue is left in the eggshell as the hatchling emerges [82].

In the 1970s, David Peakall and colleagues began developing techniques that allowed CAM tissue collected after hatching to be used as a nonlethal and noninvasive indicator of embryonic exposure to persistent environmental contaminants [206, 207]. This line of research was initially undertaken in an attempt to understand the onset and accumulation of the pesticide, 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) in peregrine falcon (*Falco peregrinus*) eggs in regards to population declines. Using museum preserved eggshells, Peakall demonstrated the absence of 2,2-bis-(*p*-chloro phenyl)-1,1-dichloroethylene (DDE), a major metabolite of DDT, in eggshell membranes collected before the production of DDT and a subsequent increase of DDE in eggshell membranes thereafter [207]. This seminal study not only revealed that contaminants

could accumulate in eggshell membranes, but also that it was possible to use a nonlethal source to gather this information. It was suggested that CAM exposure to environmental contaminants could arise internally, i.e. contaminants maternally deposited into the yolk enter circulation and are transported to the CAM or sequestered to the allantois [82], or externally, i.e. contaminants in the nest environment are transported across the eggshell and are transported to the CAM, which lines the inner surface [208, 209].

The use of eggshell membranes to estimate contaminant burdens in the egg was validated by Peakall et al. (1983) in a comparison of CAM and whole egg concentrations of DDE and polychlorobiphenyls (PCBs) in peregrine falcons [210]. Subsequent studies in great blue herons (*Ardea herodiasfannini*) [211], loggerhead sea turtles (*Caretta caretta*) [82], and the American Alligator (*Alligator mississippiensis*) [212] have all revealed a strong positive relationship between the concentrations of organochlorine contaminants in the CAM and those in the egg. Further, a laboratory study conducted in the chicken (*Gallus domesticus*) demonstrated that organochlorine contaminant concentrations in the CAM could be used to estimate contaminant load not only in the egg, but also in the laying hen. In addition, a significant relationship between CAM contaminant load and hepatic enzyme activity in chicks and hens was demonstrated indicating that CAM contaminant concentration could be used as an indicator of a biological response [80].

The chemicals mentioned above have now been demonstrated to behave as endocrine disrupting contaminants (EDCs). The EDC hypothesis, originally articulated in 1991, suggested that many man-made and naturally occurring chemicals had the ability

to disrupt the normal functioning of the endocrine system at concentrations currently found in our environment [213]. The vast majority of EDC studies have described steroid receptor-mediated interactions where an environmental contaminant interferes with function by either binding the receptor and thus, behaving as a hormone agonist or by blocking receptor binding and thereby behaving as a hormone antagonist [214, 215]. However, there is increasing evidence that EDCs can also alter steroid action through disruption of steroid hormone synthesis via modification of steroidogenic enzyme pathways and through disruption of steroid hormone metabolism via inhibition of enzymes involved in steroid elimination (as reviewed in [215]).

All EDCs are not estrogenic in their actions; however, estrogenic-mediated actions continue to dominate the field of EDC research [214]. One such xenoestrogen, Bisphenol A (BPA, 2,2-bis-(4-hydroxyphenyl)-propane) has been the subject of great interest and controversy in recent years [216]. Originally synthesized in 1891, BPA was shown to have the estrogenic properties as early as the 1930s [217]. However, BPA was passed over as a pharmaceutical estrogen [218] and instead is used today in the manufacture of polycarbonate plastics and epoxy resins. As one of the highest-volume chemicals produced worldwide, BPA is ubiquitous and widespread in our environment [219, 220] and is present in many common consumer products, such as in the lining of food and beverage cans, plastic bottles, and dental sealants [221].

Aside from identifying the presence and accumulation of environmental contaminants in the CAM, there is currently very little known concerning if and how these chemicals impact this tissue. Yet, an investigation of cytochrome P4501A-catalyzed deethylation of 7-ethoxyresorufin (EROD) activity in the CAMs of chicken

[208, 222] and Eider duck (*Somateria mollissima*) [222] revealed that EROD activity was not only present in the chicken and duck CAM, but was also induced 10-50 fold in the chick CAM following exposure to PCB 126 [208] and 5 fold in the duck CAM following exposure to β-naphthoflavone (BNF) [222]. Granberg et al. (2003) further investigated the binding of labeled polycyclic aromatic hydrocarbons (PAHs) in the chicken CAM and demonstrated that PCB 126 and BNF exposure increased PAH binding in the endothelial cells of CAM blood vessels [209]. Overall, the findings from these studies suggested that the CAM 1) expresses CYP genes important in the biotransformation of xenobiotics, 2) has the capability to biotransform environmental contaminants, and 3) could be a target of EDC action [208, 209, 222].

It has recently been demonstrated that the CAMs of chicken, American alligator and Red-belly slider turtle (*Pseudemys nelsoni*) exhibit steroid hormone synthetic activity and are targets of steroid signaling as they exhibit steroid hormone receptors ([116], Chapters 2, 3, 4). Therefore, I hypothesized that the presence of EDCs in the CAM could potentially interfere with steroid signaling and or steroidogenesis in this tissue. To investigate this hypothesis, mRNA expression was examined in the American alligator CAM following exposure to a naturally occurring estrogen, 17β-estradiol (E2) and an environmental estrogen, BPA. It has now been demonstrated that BPA behaves as an estrogen agonist by binding the estrogen receptor (ER) not only in mammalian species, but also in nonmammalian vertebrates, including chicken, the green anole lizard (*Anolis carolinensis*), the African clawed frog (*Xenopus laevis*) and the rainbow trout (*Oncorhynchus mykiss*) (reviewed in [223]) and that BPA, like E2, can stimulate the transcription of ER mRNA [224]. In addition, both E2 and BPA also have the ability

to bind the progesterone receptor (PR) [225] and induce PR mRNA transcription [226, 227]. Furthermore, E2 and BPA have been shown to affect the expression and activity of steroidogenic enzymes, but the direction of change reported is inconsistent. For example, E2 and BPA have been reported to both stimulate [228, 229] and inhibit [230-232] CYP19 (also called aromatase) gene expression or activity. In addition, E2 was reported to increase 3β-hydroxysteroid dehydrogenase activity [233, 234], while E2 and BPA were reported to decrease the expression or activity of this same enzyme [235-237]. The reported differences cited here likely reflect species, tissue/cell, and mechanism specific responses as even within human placental studies, opposite effects of E2 and BPA on CYP19 expression have been reported [228, 232].

Here, I investigated whether estrogenic exposure affects steroid hormone processes in the CAM through receptor-mediated actions by examining mRNAs coding for estrogen receptor α (ESR1), estrogen receptor β (ESR2) and PR. In addition, I examined whether CAM steroidogenic enzyme actions were affected by estrogenic exposure by examining mRNAs coding for cytochrome P450, family 19, subfamily A, polypeptide 1 (CYP19A1), and hydroxy-delta-5-steroid dehydrogenase, 3 beta-and steroid delta-isomerase 1 (HSD3B1), the key steroidogenic enzymes involved in the synthesis of estrogens and progestins; respectively [97]. Our study attempts to determine whether CAM steroid signaling and/or steroidogenesis are regulated in response to estrogenic exposure. The question of regulation is vital to the understanding of this system because if gene expression in the CAM is regulated as such, it is possible for gene expression to be altered by exposure to xenoestrogens. In addition, developmental estrogenic exposure has been demonstrated to affect the

timing of birth, as well as fetal and neonatal growth patterns in a number of organisms, such as rodents, ewes, and Japanese medaka fish (*Oryzias latipes*) [238-241]. Therefore, the effect of *in ovo* estrogenic exposure on the timing of hatch and hatchling body morphometrics of alligators was also investigated.

Materials and Methods

Egg Collection

Fieldwork was conducted under permits from the Florida Fish and Wildlife Conservation Commission and the U.S. Fish and Wildlife Service. During the summer of 2009, American alligator (*Alligator mississippiensis*) eggs were collected from nests located within Lake Woodruff National Wildlife Refuge (Deland, Florida) and were transported to the University of Florida for incubation. Within 48 h of arrival, eggs were candled to assess viability and embryos from 2 to 3 eggs per clutch were used to determine the average embryonic stage of the clutch based on criteria described by Ferguson [129]. Eggs from six clutches for gene expression analyses or five clutches for hatching analyses were evenly distributed into treatment groups and incubated at a female producing temperature of 30°C.

In Ovo Exposure

At embryonic stage 20 (immediately prior to the thermal sensitive period of sex determination), eggs were weighed to the nearest 0.05 grams and received one of the following treatments applied topically to the eggshell; 95% ethanol vehicle control (0.5 µL/g of egg weight), 17β-Estradiol (0.014µg/g of egg weight), or Bisphenol A (1.4 µg/g of egg weight). Following treatment, eggs were allowed to air dry at room temperature for 10 minutes and returned to the incubator. The timing of exposure, treatment

application, and doses of BPA and E2 are based on studies in another crocodilian, C*aiman latirostris* [242, 243].

CAM Collection, RNA Isolation, Reverse Transcription and Real-time Quantitative Polymerase Chain Reaction (RT-qPCR)

CAM samples were dissected away from the embryo and eggshell at 12, 24, 48 and 72 hours post exposure, washed in 1X phosphate buffered saline (PBS),stored in the RNA preservative, RNAlater solution (Ambion) at 4°C overnight and stored at -20°C until RNA isolation. Total RNA was isolated, reverse transcribed and complementary DNA was analyzed in triplicate as described in Chapter 3. RT-qPCR was performed on CAM samples collected at 12 hours (vehicle control n=11, BPA n=11, E2 n=12), 24 hours (vehicle control n=12, BPA n=11, E2 n=12), 48 hours (vehicle control n=12, BPA n=13, E2 n=11), and 72 hours (vehicle control n=12, BPA n=12, E2 n=12). Primer pairs were combined and diluted to a final concentration of 10 µM and are reported in Table 5-1. Triplicate data for each gene were averaged and mRNA expression levels of the steroid receptors (ESR1, ESR2, and PR) and steroidogenic enzymes (HSD3B1 and CYP19A1) were determined by the absolute quantification method [87] as previously described in Chapter 3. Controls lacking cDNA template were included on every RT-qPCR plate to determine the specificity of target cDNA. Additionally, to confirm that target cDNA was not contaminated by genomic DNA, RT-qPCR was performed with RPL8 primers on the RNA isolated from every sample. To normalize mRNA expression levels, RT-qPCR was performed on all samples with ribosomal protein L8 (RPL8). Data are reported as relative expression and represent mean normalized mRNA transcript percentage in (% of RPL8).

Hatch Data

Hatch dates were recorded for every animal and timing of hatch was calculated as the difference between embryonic stage 20 and the day of hatching. At hatching, body mass (BM), tail girth (TG), snout-vent length (SVL), and total length (TL) were measured.

Statistical Analysis

All statistical analyses were performed in the R statistical programming environment version 2.13.1 [95]. For gene expression analyses, the total numbers of mRNA transcripts for 1 internal control and 5 target genes from the CAM were determined by RT-qPCR. To quantify relative expression of target genes, I normalized mRNA expression of each sample to RPL8. I used linear mixed effects models (LMMs) to compare relative mRNA expression between the treatment groups at each discrete time period. In these analyses, *in ovo* exposure was treated as a fixed effect, and RT-qPCR plate and maternal clutch were treated as random effects; however, maternal clutch did not significantly reduce model deviance and was excluded from the model for all genes. Model assumptions were evaluated visually via examination of residuals and QQ plots and log or square-root transformations were performed when necessary to normalize errors. Outliers were identified from residuals and QQ plots and the largest value of ESR1 and ESR2 in the E2 treatment at 72 hours and the largest value of HSD3B1 in the BPA treatment at 48 hours, were removed from the study (note that inclusion of these values did not change patterns of inference, but were excluded from the final analysis because they had a disproportionate influence on mean estimates and caused violation of normality). The assumption of homogeneity of variances was met for all genes. The RT-qPCR plate set up accounts for the difference in the number of

degrees of freedom between genes. The same analytical approach described above was used to estimate the effect of *in ovo* exposure on the timing of hatch and hatchling body morphometrics. In these analyses *in ovo* exposure was treated as a fixed effect and maternal clutch was treated as a random effect.

Results

Steroid Receptor and Steroidogenic Enzyme Expression in the CAM Following Estrogenic Exposure

The effect of estrogenic exposure on the level of mRNA expression in the CAM at four discrete time periods was analyzed. The level of mRNA expression was not different among the treatment groups at any of the time periods examined for ESR2 (Figure 5-2), PR (Figure 5-3), CYP19A1 (Figure 5-4), or HSD3B1 (Figure 5-5), a summary of statistical analyses are presented in Table 5-2. Likewise, ESR1 expression was not different among the treatment groups at 12 hours, 24 hours, or 48 hours post exposure. However, ESR1 mRNA in the E2 treatment group was upregulated 1.4 fold relative to the vehicle control and 1.6 fold relative to the BPA treatment group at the 72 hour time period ($F_{2,25}$=5.21, p=0.01) (Table 5-2, Figure 5-1).

The Effect of Estrogenic Exposure on Timing of Hatch

The effect of estrogenic exposure on the timing of hatch was analyzed and it was observed that alligators exposed to BPA and E2 *in ovo* hatched earlier than those exposed to the vehicle control ($F_{2,70}$=7.4, p=0.0012) (Figure 5-6). The average time to hatch for BPA and E2 exposed animals was 45.1 ± 0.4 and 45.0 ± 0.4 days, respectively, whereas the average time to hatch in vehicle exposed animals was 46.5 ± 0.7 days (Figure 5-6).

Hatchling Body Morphometrics Following Estrogenic Exposure

Next, the effect of estrogenic exposure on body morphometrics at hatch was analyzed. Body mass ($F_{2,70}$=1.22, p=0.30), tail girth ($F_{2,70}$=0.20, p=0.82), total length ($F_{2,70}$=0.007, p=0.94), and snout-vent length ($F_{2,70}$=0.72, p=0.49) were not different among the treatment groups (Figure 5-7).

Discussion

It is well established that persistent environmental contaminants accumulate in the CAM [206] and it appears that this tissue has the capability to biotransform these compounds [208, 209, 222]. Indeed, it has even been suggested that “the CAM could represent a first line of metabolic defense against certain toxic compounds penetrating the shell” [209]. The majority of pollutants identified in CAM studies are classified as EDCs, i.e. chemicals that can interfere with the normal functioning of the endocrine system by modifying steroid hormone receptor and steroidogenic enzyme mediated actions (reviewed in .[215]. Our previous studies in the chicken, alligator and turtle indicates that the CAM has the capability to respond to steroid hormone signaling and perform steroidogenesis ([116], Chapters 2, 3, 4). Therefore, I examined whether exposure to either a naturally occurring estrogen (E2) or a synthetic estrogen (BPA) could potentially interfere with steroid signaling and or steroidogenesis in the alligator CAM. Here, I demonstrate that estrogenic exposure affected steroid receptor mRNA expression by showing that topical application of E2 resulted in upregulation of CAM ESR1.

In general, E2 upregulates ESR1, ESR2 and PR (as reviewed in [244]), and in addition has been shown to upregulate or downregulate CYP19A1 [228-231] and HSD3B1 [233-235] dependent on species and tissue/cell types. Therefore, I expected

that if the CAM was responsive to estrogenic treatment, I could potentially observe a change in mRNA expression of any of these genes following estrogenic exposure. Yet, based on previous studies and as will be explained in subsequent paragraphs, I predicted that the effect of E2 exposure on CAM ESR1 should be greater than that on ESR2 and/or PR. As there is less consistency in steroidogenic enzyme responses to E2, I predicted that transcription of these genes could be affected by exposure, but that either an upregulation or downregulation was possible and that the magnitude of change would likely be less than that of ESR1.

Our finding that E2 resulted in an upregulation of ESR1 only after 72 hours suggests that topically applied E2 did not penetrate the eggshell in sufficient concentration to elicit a response in CAM ESR1 until between the 48h and 72h collection time points. This result coupled with a complete absence of an observed effect in ESR2 and PR or the steroidogenic enzymes could suggest that; 1) the dose of E2 was only sufficient to effect ESR1 expression, 2) CAM steroid receptors exhibit a differential response to E2.or 3) the time series used was insufficient to capture a response in mRNA expression beyond that of ESR1.

It has been reported that E2 is a more potent activator of ESR1 than ESR2 [245, 246] and has higher binding affinity for ER than PR [225]. ESR1 and ESR2 have distinct expression patterns in tissue distribution studies [247] and while both were identified in the CAM, ESR1 relative expression was on average 10 orders of magnitude higher than that of ESR2, suggesting that ESR1 is the predominant subtype of ER expressed in this tissue. In addition, PR relative expression in the CAM was on average an order of magnitude lower than that of ESR1. Thus, the low dose of E2 used in our study may not

have been sufficient to induce ESR2 and PR mRNA expression. Likewise, ESR1 mRNA expression is approximately 10 and 1 order(s) of magnitude higher than that of CYP19A1 and HSD3B1, respectively, which again could explain why E2 did not induce a change in mRNA expression of the steroidogenic enzymes examined.

A differential response of ESR1 and ESR2 to E2 has been noted in other gene expression studies. In the chicken, *in ovo* E2 exposure on the first day of incubation resulted in increased ESR1 mRNA expression in male gonads on day 10 of incubation, whereas gonadal ESR2 expression was unaffected by E2 exposure in either sex [248]. Likewise, Sabo-Attwood et al. (2004) showed that large mouth bass (*Micropterus salmoides*), hepatic expression of ERs differed in response to E2, with E2 inducing a response in ESR1, but not in ESR2 [249]. Therefore, it is possible that the ERs are not regulated in the same fashion by E2 in the CAM.

It is also possible that since ESR1 was unaffected by E2 exposure until 72 hours post application that our time series was insufficient to detect a response to E2 in ESR2, PR and steroidogenic enzyme mRNAs. This might seem unlikely based on a topical application study in the slider turtle (*Trachemys scripta*) which showed that labeled E2 applied to the eggshell peaked within embryos at 25 hours after application, but could be detected as early as 2 hours after application [250]. However, the turtle and alligator eggshell are vastly different in their porous nature [45] and at this point I can only speculate on the timing and concentration of topically applied E2 that penetrated the interior of the egg (see further discussion concerning internal dose below); but my findings suggest that the temporal transfer of E2 across the eggshell was less efficient than that reported in the turtle.

While BPA has been demonstrated to exhibit estrogenic actions in numerous studies, it has been shown in competitive binding assays that BPA was 1000 to 10,000 and 1000 to 2,300 times less potent than E2 in the mammalian and non-mammalian species examined, respectively (as reviewed in [223]). This could explain why an upregulation of ESR1 in response to E2, but not BPA was observed in our study. It is important to note that the estrogenic exposures of this study were the result of a one time topical application to the eggshell and the concentrations applied to the eggshell, were considered low doses of E2 and BPA. The doses, method of delivery, and timing of exposure were based on studies in another crocodilian, C*aiman latirostris*, which demonstrated effects of estrogenic exposure in post hatching animals [242, 243]. While internal dose, i.e., the concentration of E2 or BPA penetrating the interior of the egg, was not determined in this study, previous work examining the applied versus internal dose in the marine turtle (*Chelonia mydas*) revealed that only 34% and 8% of DDE applied topically at embryonic stage 21 was found in the egg and embryo, respectively at stage 28 [251]. Thus, it is fairly safe to assume that the internal doses of E2 and BPA of our study were considerably lower than those applied externally and might account for the lack of response in ESR2, PR, CYP19A1 and HSD3B1 mRNA expression to E2 and BPA exposure and ERS1 in BPA exposure.

Prematurity, either being born too soon or too small, is a leading cause of human infant mortality and morbidity [252, 253]. In the United States, the occurrence of human preterm birth has increased more than 20% and low birth weight has increased 10% from 1990 to 2006 [254]. In analyzing the distribution of spontaneous singleton births, Davidoff et al. (2006) showed that the average gestation time decreased by a week

between 1992 and 2002 [255]. There has been much speculation on whether increased exposure to environmental contaminants, such as BPA, could play a role in the increased occurrence of preterm birth and low birth weight [253]

Diethylstilbestrol (DES), a synthetic, non-steroidal estrogen that was ironically prescribed as a treatment for preterm birth and problematic pregnancies in the 1950s and 1960s has been associated with premature birth in humans [256] [257]. DES exposure in rats was shown to affect the length of gestation and either increased or decreased gestation length dependent on the timing of exposure [241]. A number of EDCs with estrogenic potential have been correlated with preterm birth including cadmium [258], organochlorine pesticides [259, 260], PCBs [261] and BPA [262]. BPA exposure has also been observed to cause premature hatching in medaka [239]. Here, I showed that in alligators both E2 and BPA exposure accelerated timing of hatch compared to vehicle controls. While the average difference between estrogenic exposed and vehicle treated animals was small, the effect of premature hatching could have significant implications and warrants further investigation.

It has also been suggested that increased estrogenic exposure during embryonic development can negatively affect fetal growth [263]. BPA exposure has been shown to decrease fetal body weight in rats [238], birth weight in ewes [240] and hatch size in medaka [239]. However, our study did not detect an effect of estrogenic exposure on alligator hatchling body morphometrics. In addition to decreasing fetal and birth body weight, *in utero* BPA exposure has been shown to increase body weight as animals age [220]; thus, it will be important to examine whether *in ovo* estrogenic exposure influenced post hatching growth rate in the alligators from our study.

The CAM has been utilized as a nonlethal indicator of embryonic exposure to environmental contaminants in numerous oviparous bird and reptile species [206]. It is likely that CAM contaminant exposure could arise from sources both internal and external to the shelled egg. Internal exposure could arise from maternal transmission, i.e. off-loading of contaminants to the yolk [264]. Contaminants present in the yolk could then be transported to the CAM via circulation. Previous studies in the loggerhead sea turtle and American alligator have demonstrated that PCBs are present in the egg prior to CAM formation. However, these compounds are readily transported to the CAM upon its development [82, 212]. In addition, external exposure could arise in the nest environment. The close proximity of the CAM to the eggshell and it's highly vascularized nature facilitate the CAMs primary role of gas exchange with the external environment. In addition, the CAM mobilizes calcium from the eggshell to the embryo [265]. In light of this, it seems likely that contaminants, which come in contact with the eggshell, perhaps through contact with contaminated feathers or feet of brooding parents in avian nests or contaminated nesting material in reptilian nests, could be transported to the CAM.

It is evident from previous studies in the American alligator and other species with temperature sex determination, that E2 and other chemicals applied topically have the ability to penetrate the eggshell and cause embryonic sex reversal [250, 266, 267]. Very little is known concerning the extent of contaminant transmission across the eggshell in natural environments. Yet a recent laboratory study attempted to investigate the transmission of contaminants across the eggshell of a reptilian species as they would be exposed in the natural environment, i.e., through contaminated nesting material. Solla et al. incubated snapping turtle (*Chelydra serpentina*) eggs in soil

contaminated with various pesticides and found that internal transmission of pesticides increased with duration of contaminant exposure [268] suggesting that reptilian eggs incubated in natural environments likely absorb contaminants from nesting substrate throughout incubation.

To my knowledge no studies have quantified BPA in the CAM, yet BPA has been measured in placental tissues of rats [269] and humans [270]. While acute BPA exposure studies indicate that BPA is rapidly metabolized and excreted in adult animals, it has been suggested that lower metabolism during fetal development could result in decreased clearance [271]. As the CAM and chorioallantoic placenta are derived from the same extraembryonic membranes and perform similar functions during development, it thus stands to reason that the CAM could be exposed to BPA and play a role in its metabolism and excretion. This idea is supported by the finding that labeled BPA injected into the yolk of Japanese quail (*Coturnix japonica*) on day 3 of embryonic development was observed in the allantoic fluid on days 10 and 15 of incubation with more pronounced radioactive labeling on day 15 compared to day 10 [272].

The observation that ESR1 mRNA was upregulated in the CAM following *in ovo* E2 exposure in conjunction with our previous findings that CAM steroidogenic enzymes and steroid receptor mRNAs are affected by developmental timing ([116], Chapters 2, 3, 4) and incubation temperature (Chapter 2) indicate that gene expression in the oviparous CAM is regulated. While more work is needed to understand the regulation of steroid pathways in the CAM, establishing that gene expression in the oviparous CAM is regulated suggests that it is possible for gene expression to be altered in this tissue. Our study indicates that estrogenic exposure affects mRNA expression in the CAM and

thus, suggests that environmental contaminants acting through estrogenic pathways could impact CAM gene expression. Dose response studies examining steroid signaling, steroid synthesis and steroid metabolism mediated actions in response to EDCs known to bioaccumulate in this tissues are needed. Further, our previous studies have demonstrated the presence of the androgen receptor and glucocorticoid receptor in the CAM (Chapter 3, Chapter 4) and future studies should investigate whether these receptors are affected by exposure to naturally occurring or synthetic androgens and glucocorticoids. As BPA has been demonstrated to strongly bind the human estrogen-related receptor γ (ERRγ) [273], which is highly expressed in the placenta [274], I suggest that future work should also investigate if the CAM expresses EERγ and how ERRγ expression might be affected by estrogenic exposure.

Table 5-1. Alligator PCR primers used for RT-Quantitative real-time PCR

Gene	Direction	Sequence (5'-3')	Annealing (C)	GenBank accession number or reference
RPL8	Sense	GGTGTGGCTATGAATCCTGT	62.9	Katsu et al. (2004)
	Antisense	ACGACGAGCAGCAATAAGAC		
PR	Sense	AAATCCGTAGGAAGAACTGTCCAG	67.5	AB115911
	Antisense	GACCTCCAAGGACCATTCCA		
ESR1	Sense	AAGCTGCCCCTTCAACTTTTTA	66.5	AB115909
	Antisense	TGGACATCCTCTCCCTGCC		
ESR2	Sense	AAGACCAGGCGCAAAAGCT	65	AB115910
	Antisense	GCCACATTTCATCATTCCCAC		
CYP19A1	Sense	CAGCCAGTTGTGGACTTGATCA	62	AY029233
	Antisense	TTGTCCCCTTTTTCACAGGATAG		
HSD3B1	Sense	GTGATCCCATCTGCAATGGTG	60	Milnes et al. (2008)
	Antisense	CCATCTGCCTTCAGGACATGTT		

Table 5-2. Statistic summary of RT-Quantitative real-time PCR on the alligator CAM at four time periods post exposure.

Gene	12 hour[a]	24 hour[a]	48 hour[a]	72 hour[a]
ESR1	2,22=2.60, ns	2,23=0.82, ns	2,24=0.77, ns	2,24=5.21, *
ESR2	2,22=1.02, ns	2,23=0.22, ns	2,24=2.20, ns	2,24=0.21, ns
PR	2,27=2.57, ns	2,27=1.46, ns	2,28=0.16, ns	2,26=0.29, ns
CYP19A1	2,27=1.22, ns	2,27=0.16, ns	2,28=0.86, ns	2,26=0.07, ns
HSD3B1	2,27=0.38, ns	2,27=0.12, ns	2,27=0.63, ns	2,26=0.69, ns

[a] Data presented as F(df)=, p-value=

** p<0.01

* p<0.05

ns=not significant

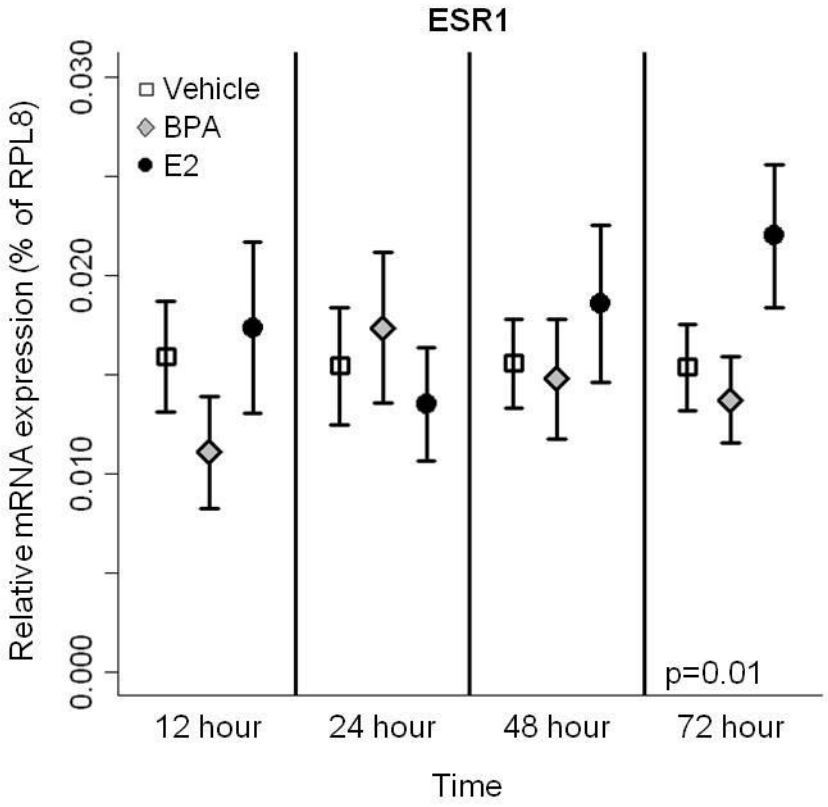


Figure 5-1. Relative mRNA expression of ESR1 in the CAM following estrogenic exposure. RT-qPCR analysis of mRNA coding for ESR1 at 12, 24, 48, and 72 hours following topical application of 95 % ethanol vehicle control (open squares), BPA (grey diamonds), or E2 (solid circles). Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RPL8) ± SEM.

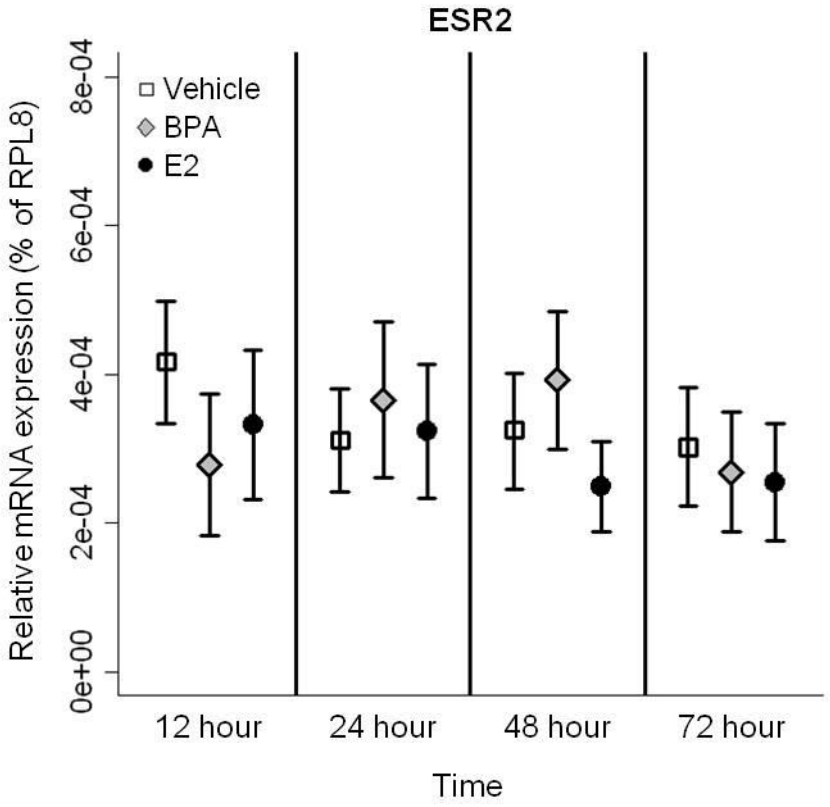


Figure 5-2. Relative mRNA expression of ESR2 in the CAM following estrogenic exposure. RT-qPCR analysis of mRNA coding for ESR2 at 12, 24, 48, and 72 hours following topical application of 95 % ethanol vehicle control (open squares), BPA (grey diamonds), or E2 (solid circles). Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RPL8) ± SEM.

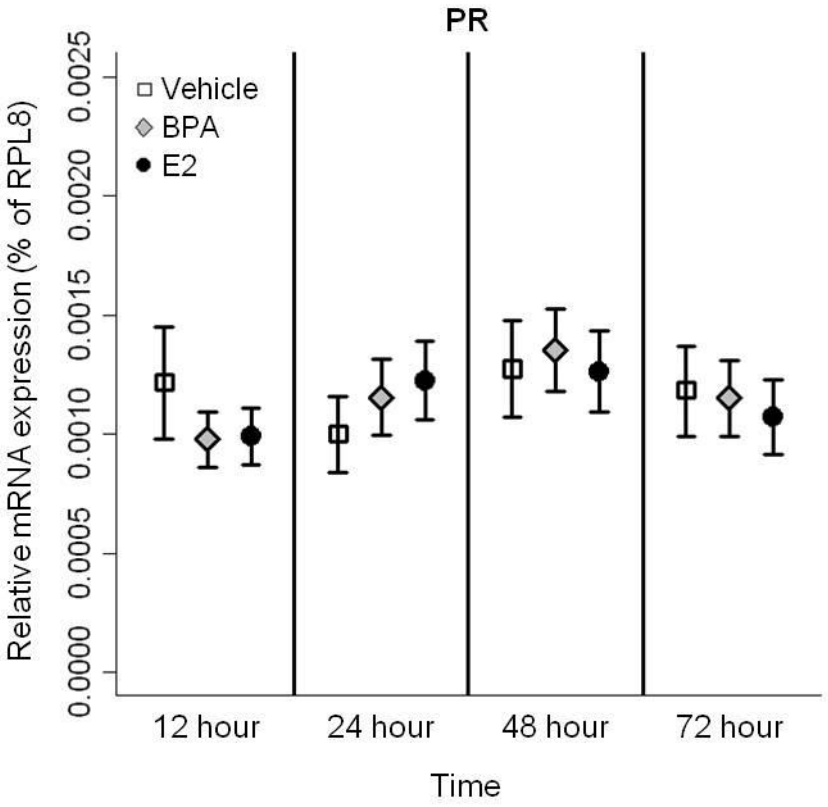


Figure 5-3. Relative mRNA expression of PR in the CAM following estrogenic exposure. RT-qPCR analysis of mRNA coding for PR at 12, 24, 48, and 72 hours following topical application of 95 % ethanol vehicle control (open squares), BPA (grey diamonds), or E2 (solid circles). Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RPL8) ± SEM.

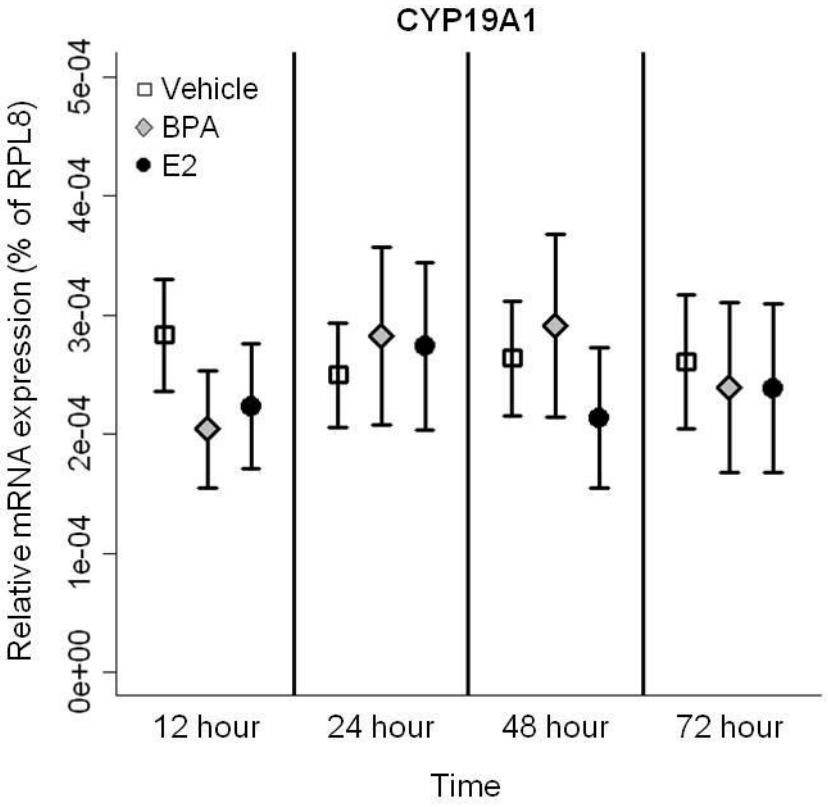


Figure 5-4. Relative mRNA expression of CYP19A1 in the CAM following estrogenic exposure. RT-qPCR analysis of mRNA coding for CYP19A1 at 12, 24, 48, and 72 hours following topical application of 95 % ethanol vehicle control (open squares), BPA (grey diamonds), or E2 (solid circles). Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RPL8) ± SEM.

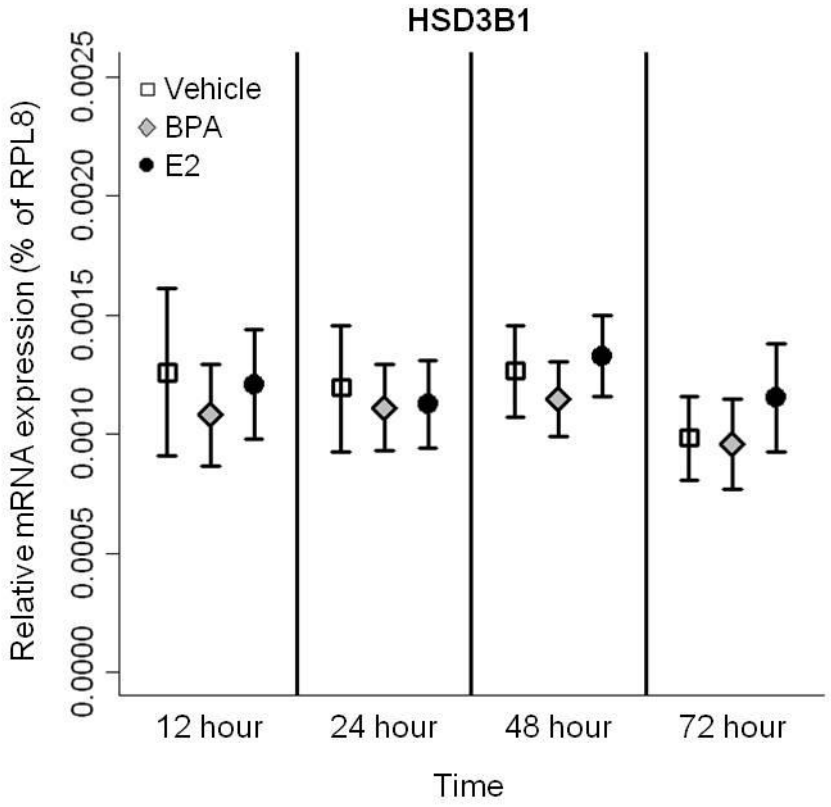


Figure 5-5. Relative mRNA expression of HSD3B1 in the CAM following estrogenic exposure. RT-qPCR analysis of mRNA coding for HSD3B1at 12, 24, 48, and 72 hours following topical application of 95 % ethanol vehicle control (open squares), BPA (grey diamonds), or E2 (solid circles). Data are reported as relative mRNA expression and represent mean normalized mRNA transcript percentage in (% of RPL8) ± SEM.

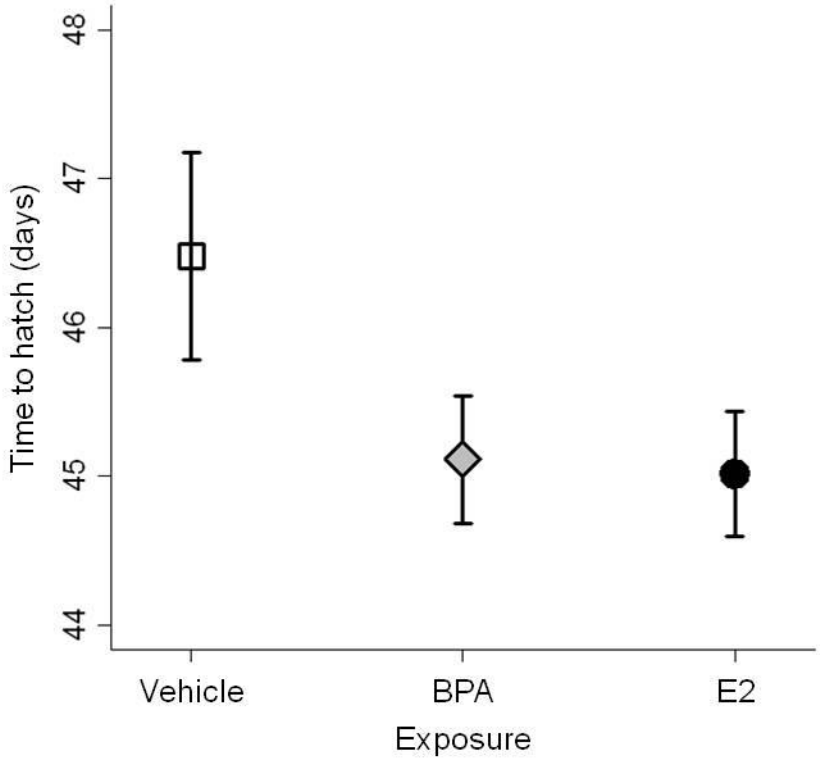


Figure 5-6. The effect of estrogenic exposure on timing of hatch. Timing of hatch was recorded for animals exposed *in ovo* at stage 20 to either 95% ethanol vehicle control (open squares), BPA (grey diamonds), or E2 (solid circles). Timing of hatch was calculated as the difference between embryonic stage 20 and day of hatching. Data represent mean hatch day ± SEM.

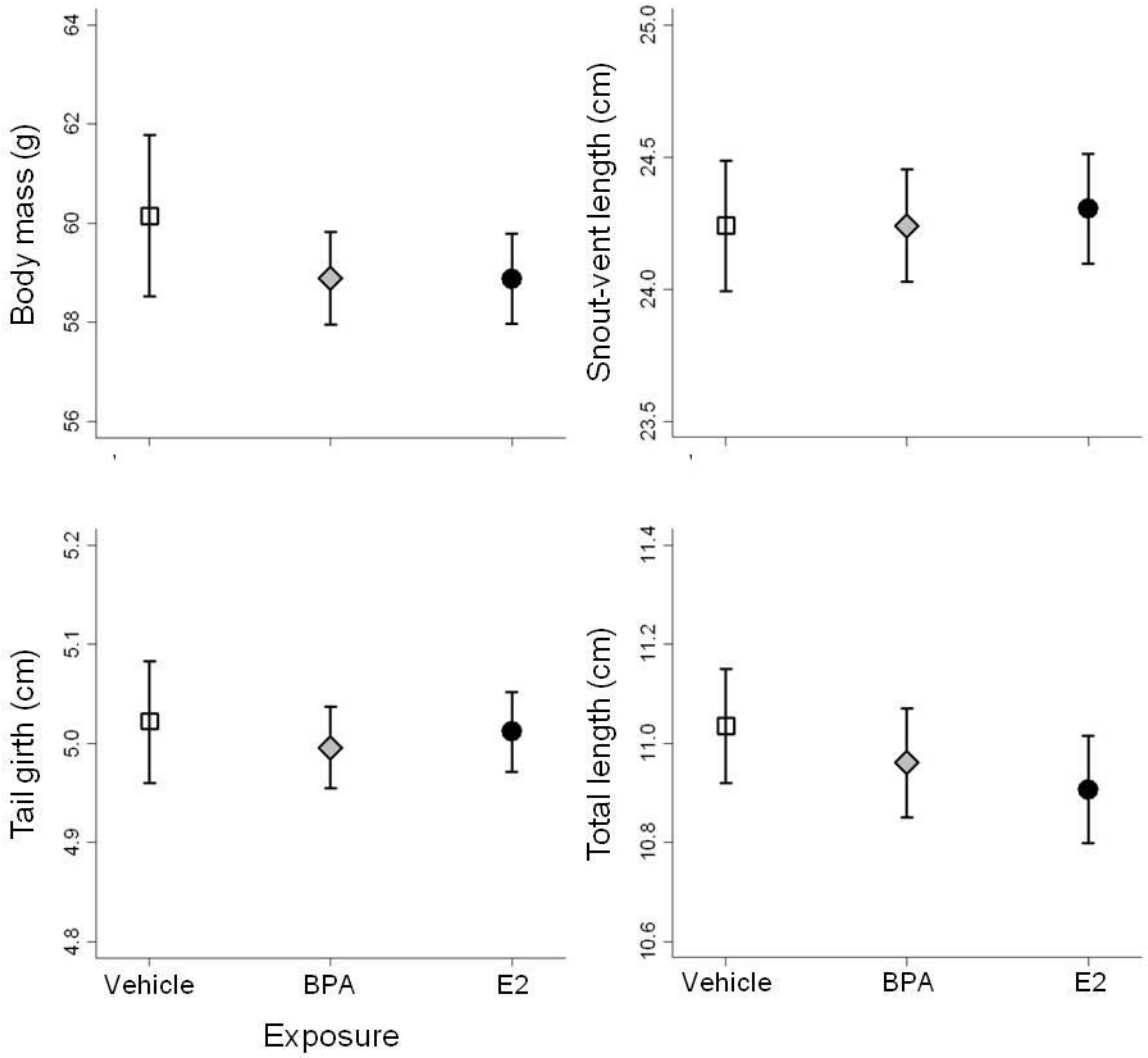


Figure 5-7. The effect of estrogenic exposure on hatchling body morphometrics. Body mass, tail girth, snout-vent length and total length was recorded upon hatching for animals exposed *in ovo* at stage 20 to either 95 % ethanol vehicle control (open squares), BPA (grey diamonds), or E2 (solid circles). Data represent mean values ± SEM.

CHAPTER 5
SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Summary and Conclusions

While the placenta was first proposed to function as an endocrine organ in the early 1900s [5], the role of steroid hormone synthesis, metabolism and signaling continues to be defined in the placenta today [275-277], and the evolution of these functions in the placenta remains as a fundamental research question [278]. Prior to my work, it was known that the embryonic contribution to the mammalian placenta performs steroid hormone synthesis and responds to steroid hormone signaling via steroid receptors [113, 279-283] (Figure 6-1). In addition, the ability to perform steroid hormone synthesis had been demonstrated in the embryonic contribution to the squamate placenta in one species of viviparous lizard [21] (Figure 6-1). As the embryonic contributions to mammalian and squamate placentae, i.e. the extraembryonic membranes, are also present in birds and oviparous reptiles, I looked to the precursor of the amniote placenta in an attempt to understand its evolution of endocrine function.

Results from Chapter 2 showed that the chicken chorioallantoic membrane (CAM) expressed mRNA coding for steroidogenic enzymes required for steroid biosynthesis, had the ability to perform progesterone (P4) synthesis, and showed mRNA expression of estrogen receptor α (ESR1) and mRNA and protein level expression of the progesterone receptor (PR). This represented the first examination of steroid activity in the CAM of an oviparous amniote and demonstrated that steroidogenesis and steroid signaling in extraembryonic membranes are not exclusive characteristics of placental mammals and squamates. Although, the oviparous CAM of birds might have proven unique in this regard, we hypothesized that CAM steroid activity would extend to other

oviparous species, thus representing an additional unifying characteristic of amniotes and a possible explanation for how the placenta evolved as an endocrine organ.

In Chapter 3 and Chapter 4, I investigated this hypothesis in the CAMs of other oviparous amniotes, the American alligator and Florida red-belly slider turtle, respectively. Chapter 3 showed that the alligator CAM expressed mRNA coding for some of the steroidogenic enzymes identified in the chicken as well as mRNA and protein level expression of PR. In addition, Chapter 3 identified a regulator of steroidogenesis (NR5A1 also called SF-1), the steroidogenic enzyme (CYP19A1 also called aromatase) and estrogen receptor β (ESR2), androgen receptor (AR) and glucocorticoid receptor mRNAs and ESR1 protein for the first time in an oviparous reptile CAM. Chapter 4 revealed that the turtle CAM also expressed mRNA coding for ESR1, ESR2 and AR and mRNA and protein level expression of the PR. In addition, Chapter 4 showed that the turtle CAM, like the chicken, had the ability to perform P4 synthesis.

Collectively, results from Chapters 2 through Chapter 4 showed that the CAMs of species representing three branches of the extant oviparous amniote phylogeny had the capability to perform steroidogenesis and respond to steroid hormone signaling (Figure 6-1) and supports our hypothesis that these features could be a conserved trait of amniote extraembryonic membranes, regardless of reproductive mode. If steroidogenesis and steroid signaling in extraembryonic membranes is conserved, this would then suggest that the endocrine role of the amniote placenta likely evolved initially in an oviparous ancestor and offers a new hypothesis for the evolution of an endocrine placenta.

In Chapter 2 through Chapter 4, the mRNA expression of some genes was observed to change during development. In addition, Chapter 3 showed that mRNA expression of some genes was affected by incubation temperature. These results suggested that the expression of genes involved in steroid synthesis and signaling could be regulated in this tissue. Chapter 5 attempted to determine if CAM steroid receptors and/or steroidogenic enzymes were regulated in response to estrogenic exposure. Chapter 5 showed that a one time, low dose of the naturally occurring estrogen, 17β-estradiol, applied topically to the alligator eggshell resulted in an upregulation of CAM ESR1. While an effect on CAM gene expression was not observed in response to the synthetic estrogen, Bisphenol A, results from Chapter 5 nonetheless suggested that steroid activity in the CAM, which is known to accumulate environmental contaminants [206] could be affected by exposure to xenoestrogens.

In squamates, the transition from oviparity to viviparity involves changes in the timing of egg retention, thickness of the eggshell, and the development of a placenta [14]. With the over 100 independent evolutionary origins of viviparity in squamate reptiles [7, 8], it seems equally likely that the transition in reproductive mode; 1) may not be an exceedingly difficult one to make and 2) may occur in response to various selective forces, but likely involves similar molecular, cellular and morphogenic pathways. Therefore, we should not be surprised to find that many of the required molecular, cellular and organ level characteristics of viviparity, including endocrine signaling, were already present in the oviparous ancestors allowing them to be modified for a viviparous mode of reproduction. However, differences in the use of various

pathways and mechanisms and timing associated with these processes would likely occur.

From the previous work described in Chapter 1 and my work presented here, I suggest the following to have evolved concurrently with live-birth and consider them requirements of viviparity; intrauterine retention of the egg for the duration of embryonic development, reduction of the eggshell, and formation of a fully functioning placenta that includes not only the exchange of gases, waste and nutrients, but also endocrine signals as well. In light of my findings, it is likely that embryonic endocrine signals from the extraembryonic membranes play a key role in the maternal recognition, establishment, and maintenance of gestation in viviparous squamates. Given that the CAM is present in all birds, reptiles, and mammals, why has viviparity not evolved in birds or in the other reptiles (tuatara, crocodilians and turtles)? Simply put, the presence of a steroidogenic CAM is not enough in itself to facilitate a transition from oviparity to viviparity in the absence of extended egg retention in conjunction with continued embryonic development and decreased eggshell thickness. Egg retention is brief in birds and crocodilians, whereas egg retention is extended in some species of turtles and in the tuatara [53]. However, embryonic development is arrested in turtles and presumed to be so in the tuatara as little embryonic development occurs *in utero* and embryos are at the gastrula stage at the time of oviposition [53]. Thus, oviposition occurs at early stages of development prior to development of the CAM in birds, crocodilians, turtles, and the tuatara [53, 284]. In contrast, oviparous squamates typically retain eggs *in utero* past the time of CAM development, on average for one-third to one-half of embryonic development [284, 285]. Moreover, if the thickness of the

eggshell is not reduced *in utero*, then embryonic or extraembryonic signals that might play a role in further extending egg retention will not be in communication with the maternal uterus.

Future Directions

We currently understand much about the morphological and physiological attributes of viviparity, but despite a century of inquiry, many major questions remain. The molecular mechanisms involved in the suite of physiological (especially endocrine), anatomical, and developmental modifications associated with the evolution of viviparity have only begun to be explored. Determining the extent of steroid hormone synthesis, metabolism and signaling in the oviparous extraembryonic membranes, specifically the CAM and yolk sac membrane (YSM) could shed light on the physiological mechanisms associated with the evolution of viviparity.

In this regard, a full investigation of the CAMs ability to synthesize steroid hormones, beyond P4 is needed. In addition to P4, the synthesis and metabolism of estrogens, androgens and glucocorticoids hormones play pivotal roles in the placenta during pregnancy [111]. Therefore, establishing the ability to synthesize and metabolize these hormones not only in the CAM, but also in the YSM would greatly increase our understanding of the role of steroid hormones in oviparous extraembryonic membranes. The ability of the chicken and turtle CAM to synthesize P4 from cholesterol indicates that CYP11A1 and HSD3B1 are functional in this tissue; however, demonstrating protein expression of steroidogenic enzymes is needed. Furthermore, determining the correlation between;1) mRNA and protein levels of expression of steroidogenic enzymes and steroid receptors and 2) steroidogenic enzyme and steroid receptor levels

of protein expression is needed to understand steroid hormone mechanisms of action in these tissues.

We have speculated that steroid hormones in the CAM might regulate gene expression, influence differentiation, development, growth and vascularization of the CAM as well as play a potential role in the maintenance of embryonic development, timing of hatch and growth of the embryo. While results from Chapter 5 showed an effect of estrogenic exposure on CAM ESR1 expression and timing of hatch, demonstrating more than a correlation between these two observations will likely prove difficult. However, examining gene expression and morphological effects of *in ovo* exposure to multiple doses of naturally occurring and synthetic hormones across multiple developmental time points would help define the function of steroid hormone synthesis and signaling in the CAM.

In addition to steroid hormones, the embryonic contribution to the placenta synthesizes a suite of peptide hormones, neurohormones, growth factors and cytokines that modulate placental hormone production and release through endocrine, paracrine and autocrine mechanisms [71]. Many of these factors, such as activin [286], prolactin, growth hormone, placental lactogen [287], transforming growth factor-β, platelet-derived growth factor, fibroblast growth factor, epidermal growth factor (as reviewed in [189]), interleukin-6 [288], and prostaglandin E [289] have all been shown to affect angiogenesis in the chicken CAM. This suggests that the oviparous CAM has the mechanisms in place to respond to many important modulators of placental chemical signaling pathways. Therefore, determining how these factors might also modulate

hormone production and release in the oviparous extraembryonic membranes will be important in future studies.

Finally and most importantly, future studies must continue to examine steroidogenic and steroid signaling capabilities in the extraembryonic membranes of other oviparous amniotes to either support or refute our hypothesis that this is a conserved characteristic of amniotes (Figure 6-1). High priority should be given to examining the CAM and YSM of oviparous lizards, snakes and mammals, and to comparative studies of steroidogenic and steroid signaling capabilities of these tissues between closely related oviparous and viviparous squamate species. Further, should our hypothesis be supported through future research efforts it will be important to investigate steroid activity in the YSM of fishes and amphibians as this is the ancestral extraembryonic membrane of vertebrates and the only extraembryonic membrane of anamniotes.

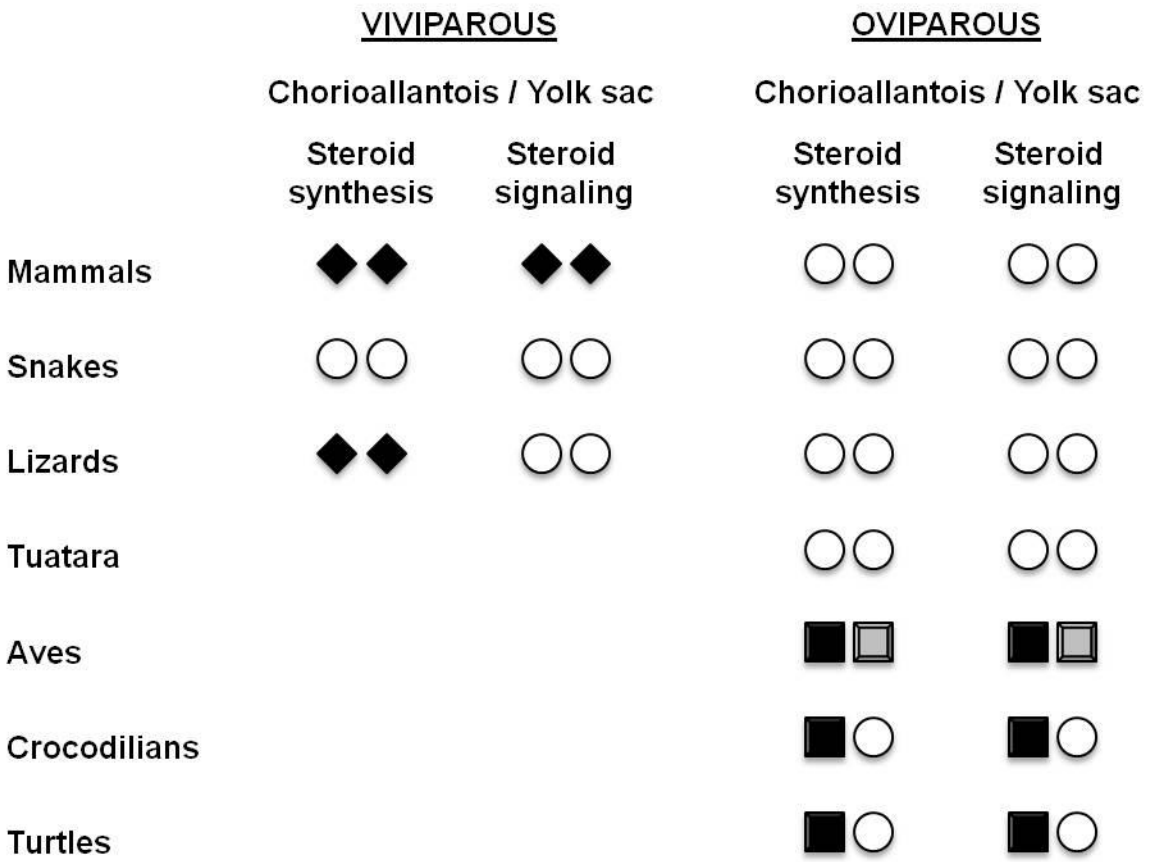


Figure 6-1. Evidence of endocrine extraembryonic membranes in amniotes. Previous studies (black diamonds) demonstrated that the embryonic contributions to the chorioallantoic and yolk sac placenta have the ability to perform steroid synthesis and respond to steroid hormone signaling in the viviparous taxa noted The work presented here (black squares) provided evidence that the oviparous CAM of the taxa noted shares that capability. In addition, I have recently observed steroidogenesis and steroid signaling capabilities in the YSM of chicken (grey squares, data not shown here). Open circles denote current holes in our understanding of this system and where future research efforts are needed.

LIST OF REFERENCES

1. Mess A, Blackburn DG, Zeller U. Evolutionary transformations of fetal membranes and reproductive strategies. J Exp Zool A 2003; 299A:3-12.

2. Aristotle, Platt A. *De Generatione Animalium*. In: Smith JA, Ross WD (eds.), The works of Aristotle translated into English, vol. 5. Oxford: Clarendon Press 1912.

3. Wourms JP, Lombardi J. Reflections on the evolution of piscine viviparity. Am Zool 1992; 32:276-293.

4. Stewart JR, Thompson MB. Evolution of placentation among squamate reptiles: recent research and future directions. Comp Biochem Physiol, A 2000; 127:411-431.

5. Del Mar Yllera M, Alexandre-Pires GM, Cifuentes JM. Placenta: regularization of neovascularization. Microvascularization pattern of the rabbit term placenta. Microsc Res Tech 2003; 60:38–45.

6. Diczfalusy E. My life with the fetal-placental unit. Am J Obstet Gynecol 2005; 193:2025-2029.

7. Shine R. The evolution of viviparity in reptiles: an ecological analysis. In: Gans C, Billett F (eds.), Biology of the reptilia, vol. 15. New York: John Wiley & Sons; 1985: 605-694.

8. Blackburn DG. Are viviparity and egg-guarding evolutionarily labile in squamates? Herpetologica 1999; 55:556-573.

9. Dulvy NK, Reynolds JD. Evolutionary transitions among egg-laying, live-bearing and maternal inputs in sharks and rays. Proc R Soc Lond [Biol] 1997; 264:1309-1315.

10. Goodwin NB, Dulvy NK, Reynolds JD. Life-history correlates of the evolution of live bearing in fishes. Philos Trans R Soc Lond, B 2002; 357:259-267.

11. Gower DJ, Giri V, Dharne MS, Shouche YS. Frequency of independent origins of viviparity among caecilians (Gymnophiona): evidence from the first 'live-bearing' Asian amphibian. J Exp Biol 2008; 21:1220-1226.

12. Reynolds JD, Goodwin NB, Freckleton RP. Evolutionary transitions in parental care and live bearing in vertebrates. Philos Trans R Soc Lond, B 2002; 357:269-281.

13. Zeller U. Mammalian reproduction: Origin and evolutionary transformations. Zool Anz 1999; 238:117-130.

14. Guillette LJ, Jr. The evolution of viviparity in lizards. Bioscience 1993; 43:742-751.

15. Saidapur SK. Structure and function of postovulatory follicles (corpora lutea): the ovaries of nonmammalian vertebrate. Int Rev Cytol 1982; 75:243–285.

16. Rothchild I. The yolkless egg and the evolution of eutherian viviparity. Biol Reprod 2003; 68:337-357.

17. Senger P. Pathways to pregnancy and parturition. Pullman, WA: Current Conceptions, Inc; 2005.

18. Koob TJ, Callard IP. Reproductive endocrinology of female elasmobranchs: lessons from the little skate (*Raja erinacea*) and spiny dogfish (*Squalus acanthias*). J Exp Zool 1999; 284:557-574.

19. Powell ML, Kavanaugh S, Sower SA. Identification of a functional corpus luteum in the Atlantic hagfish, *Myxine glutinosa*. Gen Comp Endocrinol 2006; 148:95-101.

20. Callard IP, Fileti LA, Perez LE, Sorbera LA, Giannoukos G, Klosterman LL, Tsang P, McCracken JA. Role of the corpus luteum and progesterone in the evolution of vertebrate viviparity. Am Zool 1992; 32:264-275.

21. Girling JE, Jones SM. In vitro progesterone production by maternal and embryonic tissues during gestation in the southern snow skink (*Niveoscincus microlepidotus*). Gen Comp Endocrinol 2003; 133:100-108.

22. Arslan M, Zaidi P, Lobo J, Zaidi AA, Qazi MH. Steroid levels in preovulatory and gravid lizards (*Uromastix hardwicki*). Gen Comp Endocrinol 1978; 34:300-303.

23. Gobbetti A, Zerani M, DiFiore MM, Botte V. PGF2α, PGE2, progesterone, and estradiol-17β, secretion by the corpus luteum of the oviparous lizard, *Podarcis sicula sicula*. In vitro studies. Prostaglandins 1993; 45:159-166.

24. Niswender GD, Juengel JL, McGuire WJ, Belfiore CJ, Wiltbank MC. Luteal function: the estrous cycle and early pregnancy. Biol Reprod 1994; 50:239-247.

25. Simpson ER, MacDonald PC. Endocrine physiology of the placenta. Annu Rev Physiol 1981; 43:163-188.

26. Spencer TE, Johnson GA, Burghardt RC, Bazer FW. Progesterone and placental hormone actions on the uterus: Insights from domestic animals. Biol Reprod 2004; 71:2-10.

27. Spencer T, Bazer F. Conceptus signals for establishment and maintenance of pregnancy. Reprod Biol Endocrinol 2004; 2:49.

28. Custodia-Lora N, Callard IP. Progesterone and progesterone receptors in reptiles. Gen Comp Endocrinol 2002; 127:1-7.

29. Guillette LJ, Jr., Cree A. Morphological changes in the corpus luteum of tuatara (*Sphenodon punctatus*) during gravidity. J Morphol 1997; 232:79-91.

30. Guillette LJ, Jr., Woodward AR, You-Xiang Q, Cox MC, Matter JM, Gross TS. Formation and regression of the corpus luteum of the American alligator (*Alligator mississippiensis*). J Morphol 1995; 224:97-110.

31. Cruz MVS, Mendez-de la Cruz FR. Corpus luteum through the gestation of *Sceloporus palaciosi* (Sauria : Phrynosomatidae). Copeia 1999:214-218.

32. Guarino FM, Paulesu L, Cardone A, Bellini L, Ghiara G, Angelini F. Endocrine activity of the corpus luteum and placenta during pregnancy in *Chalcides chalcides* (Reptilia, Squamata). Gen Comp Endocrinol 1998; 111:261-270.

33. Martinez-Torres M, Hernandez-Caballero ME, Alvarez-Rodriguez C, Luis-Diaz JA, Ortiz-Lopez G. Luteal development and progesterone levels during pregnancy of the viviparous temperate lizard *Barisia imbricata imbricata* (Reptilia: Anguidae). Gen Comp Endocrinol 2003; 132:55-65.

34. Uribe MDA, Omana MEM, Quintero JEG, Guillette LJ, Jr. Seasonal-variation in ovarian histology of the viviparous lizard *Sceloporus torquatus torquatus*. J Morphol 1995; 226:103-119.

35. Jones RE, Guillette LJ, Jr. Hormonal-control of oviposition and parturition in lizards. Herpetologica 1982; 38:80-93.

36. Albergotti LC, Guillette LJ, Jr. Viviparity in reptiles: evolution and endocrine physiology. In: Norris DO, Lopez KH (eds.), Hormones and reproduction in vertebrates, vol. 3: Elsevier; 2011: 247-275.

37. Jones SM, Swain R. Annual reproductive cycle and annual cycles of reproductive hormones in plasma of female *Niveoscincus metallicus* (Scincidae) from Tasmania. J Herpetol 1996; 30:140-146.

38. Fergusson B, Bradshaw SD. Plasma arginine vasotocin, progesterone, and luteal development during pregnancy in the viviparous lizard *Tiliqua rugosa*. Gen Comp Endocrinol 1991; 82:140-151.

39. Highfill DR, Mead RA. Sources and levels of progesterone during pregnancy in the garter snake, *Thamnophis elegans*. Gen Comp Endocrinol 1975; 27:389-400.

40. Chan SWC, Ziegel S, Callard IP. Plasma progesterone in snakes. Comp Biochem Physiol, A 1973; 44:631-637.

41. Guillette LJ, Jr., Spielvogel S, Moore FL. Luteal development, placentation, and plasma progesterone concentration in the viviparous lizard *Sceloporus jarrovi*. Gen Comp Endocrinol 1981; 43:20-29.

42. Guillette LJ, Jr. The evolution of vertebrate viviparity: morphological modifications and endocrine control. In: Wake DB, Roth G (eds.), Complex organismal functions: integration and evolution in vertebrates, Dahlem workshop report #45. Chichester: J. Wiley and Sons Ltd; 1989: 219-233.

43. Guillette LJ, Jr. The evolution of viviparity in amniote vertebrates - new insights, new questions. J Zool 1991; 223:521-526.

44. Heulin B, Stewart JR, Surget-Groba Y, Bellaud P, Jouan F, Lancien G, Deunff J. Development of the uterine shell glands during the preovulatory and early gestation periods in oviparous and viviparous *Lacerta vivipara*. J Morphol 2005; 266:80-93.

45. Packard MJ, Packard GC, Boardman TJ. Structure of eggshells and water relations of reptilian eggs. Herpetologica 1982; 38:136-155.

46. Palmer BD, Guillette LJ, Jr. Oviductal proteins and their influence on embryonic development in birds and reptiles. In: Deeming DC, Ferguson MWJ (eds.), Egg incubation: its effects on embryonic development in birds and reptiles. Cambridge: Cambridge Univ. Press; 1991: 29-46.

47. Qualls CP. Influence of the evolution of viviparity on eggshell morphology in the lizard, *Lerista bougainvillii*. J Morphol 1996; 228:119-125.

48. Guillette LJ, Jr., Jones RE. Ovarian, oviductal, and placental morphology of the reproductively bimodal lizard, *Sceloporus aeneus*. J Morphol 1985; 184:85-98.

49. Guillette LJ, Jr. The evolution of viviparity in fishes, amphibians, and reptiles: An endocrine perspective. In: Norris DO, Jones RE (eds.), Hormones and reproduction in fishes, amphibians, and reptiles. Plenum Press: NY; 1987: 523-562.

50. Guillette LJ, Jr. The evolution of viviparity and placentation in the high elevation, Mexican lizard *Sceloporus aeneus*. Herpetologica 1982; 38:94-103.

51. Arrayago MJ, Bea A, Heulin B. Hybridization experiment between oviparous and viviparous strains of *Lacerta vivipara*: A new insight into the evolution of viviparity in reptiles. Herpetologica 1996; 52:333-342.

52. Heulin B, Arrayago MJ, Bea A, Brana F. Characteristcs of egshells of the (oviparous X viviparous) hybrid strain of the lizard *Lacerta vivipara*. Can J Zool-Rev Can Zool 1992; 70:2242-2246.

53. Andrews RM, Mathies T. Natural history of reptilian development: constraints on the evolution of viviparity. Bioscience 2000; 50:227-238.

54. Herbert JF, Lindsay LA, Murphy CR, Thompson MB. Calcium transport across the uterine epithelium of pregnant lizards. In: Herptol. Monogr.; 2006: 205-211.

55. Kluge AG. Chordate structure and function. New York: Macmillan Publishing Co., Inc.; 1977.

56. Mossman HW. Vertebrate fetal membranes: comparative ontogeny and morphology, evolution, phylogenetic significance, basic functions, research opportunities. New Brunswick, N.J.: Rutgers University Press; 1987.

57. Stewart JR, Thompson MB. Evolutionary transformations of the fetal membranes of viviparous reptiles: a case study of two lineages. J Exp Zoo A 2003; 299A 13-32.

58. Stewart JR, Blackburn DG. Reptilian placentation: structural diversity and terminology. Copeia 1988:839-852.

59. Thompson MB, Adams SM, Herbert JF, Biazik JM, Murphy CR. Placental function in lizards. International Congress Series 2004; 1275:218-225.

60. Weekes HC. A review of placentation among reptiles with particular regard to the function and evolution of the placenta. Proc Zool Soc London 1935; 1935:625-645.

61. Blackburn DG, Callard IP. Morphogenesis of placental membranes in the viviparous, placentotrophic lizard *Chalcides chalcides* (Squamata: Scincidae). J Morphol 1997; 232:35-55.

62. Blackburn DG. Chorioallantoic placentation in squamate reptiles - structure, function, development, and evolution. J Exp Zool 1993; 266:414-430.

63. Thompson MB, Speake BK. Energy and nutrient utilisation by embryonic reptiles. Comp Biochem Physiol, A 2002; 133:529-538.

64. Adams SM, Biazik JM, Thompson MB, Murphy CR. Cyto-epitheliochorial placenta of the viviparous lizard *Pseudemoia entrecasteauxii*: a new placental morphotype. J Morphol 2005; 264:264-276.

65. Jerez A, Ramirez-Pinilla MP. The allantoplacenta of *Mabuya mabouya* (Sauria, Scincidae). J Morphol 2001; 249:132-146.

66. Thompson MB, Speake BK, Russell KJ, McCartney RJ. Nutrient uptake by embryos of the Australian viviparous lizard *Eulamprus tympanum*. Physiol Biochem Zool 2001; 74:560-567.

67. Swain R, Jones SM. Maternal-fetal transfer of H-3-labelled leucine in the viviparous lizard *Niveoscincus metallicus* (Scincidae: Lygosominae). J Exp Zool 1997; 277:139-145.

68. Speake BK, Herbert JF, Thompson MB. Evidence for placental transfer of lipids during gestation in the viviparous lizard, *Pseudemoia entrecasteauxii*. Comp Biochem Physiol, A 2004; 139:213-220.

69. Thompson MB, Stewart JR, Speake BK. Comparison of nutrient transport across the placenta of lizards differing in placental complexity. Comp Biochem Physiol, A 2000; 127:469-479.

70. Blackburn DG, Vitt LJ, Beuchat CA. Eutherian-like reproductive specializations in a viviparous reptile. Proc Natl Acad Sci USA 1984; 81:4860-4863.

71. Petraglia F, Florio P, Nappi C, Genazzani AR. Peptide signaling in human placenta and membranes: autocrine, paracrine, and endocrine mechanisms. Endocr Rev 1996; 17:156-186.

72. Henson MC. Pregnancy maintenance and the regulation of placental progesterone biosynthesis in the baboon. Hum Reprod Update 1998; 4:389-405.

73. Mesiano S, Chen EC, Fitter JT, Kwek K, Yeo G, Smith R. Progesterone withdrawal and estrogen activation in human parturition are coordinated by progesterone receptor A expression in the myometrium. J Clin Endocrinol Metab 2002; 87:2924-2930.

74. Mark PJ, Smith JT, Waddell BJ. Placental and fetal growth retardation following partial progesterone withdrawal in rat pregnancy. Placenta 2006; 27:208-214.

75. Jojovic M, Wolf F, Mangold U. Epidermal growth factor, vascular endothelial growth factor and progesterone promote placental development in rat whole-embryo culture. Anat Embryol (Berl) 1998; 198:133-139.

76. Ogle TF, Mills TM, Costoff A. Progesterone maintenance of the placental progesterone receptor and placental growth in ovariectomized rats. Biol Reprod 1990; 43:276-284.

77. Painter DL, Moore MC. Steroid hormone metabolism by the chorioallantoic placenta of the mountain spiny lizard *Sceloporus jarrovi* as a possible mechanism for buffering maternal-fetal hormone exchange. Physiol Biochem Zool 2005; 78:364-372.

78. Giannoulias D, Haluska GJ, Gravett MG, Sadowsky DW, Challis JRG, Novy MJ. Localization of prostaglandin H synthase, prostaglandin dehydrogenase, corticotropin releasing hormone and glucocorticoid receptor in rhesus monkey fetal membranes with labor and in the presence of infection. Placenta 2005; 26:289-297.

79. Pasqualini JR. Enzymes involved in the formation and transformation of steroid hormones in the fetal and placental compartments. J Steroid Biochem Mol Biol 2005; 97:401-415.

80. Bargar TA, Scott GI, Cobb GP. Chorioallantoic membranes indicate avian exposure and biomarker responses to environmental contaminants: a laboratory study with white leghorn chickens (*Gallus domesticus*). Environ Sci Technol 2003; 37:256-260.

81. Bargar TA, Sills-McMurry C, Dickerson RL, Rhodes WE, Cobb GP. Relative distribution of polychlorinated biphenyls among tissues of neonatal American alligators (*Alligator mississippiensis*). Arch Environ Contam Toxicol 1999; 37:364-368.

82. Cobb GP, Wood PD. PCB Concentrations in eggs and chorioallantoic membranes of loggerhead sea turtles (*Caretta caretta*) from the Cape Romain National Wildlife Refuge. Chemosphere 1997; 34:539-549.

83. Cross JC, Baczyk D, Dobric N, Hemberger M, Hughes M, Simmons DG, Yamamoto H, Kingdom JCP. Genes, development and evolution of the placenta. Placenta 2003:123-130.

84. Norris DO. Vertebrate endocrinology. San Diego: Academic Press; 2007.

85. McEwen RS. Vertebrate embryology. New York: Henry Holt and Company, Inc.; 1957.

86. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. J Morphol 1951; 88:49-92.

87. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J Mol Endocrinol 2000; 25:169-193.

88. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3:1-11.

89. Rozen S, Skaletsky HJ. Primer3 on the WWW for general users and for biologist programmers. Totowa, NJ: Humana Press; 2000.

90. Fridolfsson AK, Ellegren H. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol 1999; 30:116-121.

91. King SR, Matassa AA, White EK, Walsh LP, Rao RM, Stocco DM, Reyland ME. Oxysterols regulate expression of the steroidogenic acute regulatory protein. J Mol Endocrinol 2004; 32:507-517.

92. McCoy KA, Bortnick LJ, Campbell CM, Hamlin HJ, Guillette L.J. J, St. Mary CM. Agriculture alters gonadal form and function in the toad *Bufo marinus*. Environ Health Perspect 2008; 116:7.

93. Gonzalez-Moran G, Camacho-Arroyo I. Changes in the presence of progesterone receptor isoforms in the oviduct magnum of newly-hatched chicks after gonadotropins treatment. Life Sci 2003; 73:871-882.

94. González-Morán G, García-Izquierdo J, Camacho-Arroyo I. Progesterone receptor isoforms in ovary of newly-hatched chick after gonadotropin treatment during embryonic development. Comp Biochem Physiol, A 2002; 132:519-526.

95. Team RDC. R: A language and environment for statistical computing. R Foundation for Statistical Computing. In. Vienna, Austria; 2008.

96. Pinheiro JC, and Bates, D.M. Mixed effects models in S and S-Plus. Springer; 2000.

97. Payne AH, Hales DB. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr Rev 2004; 25:947-970.

98. Challis JRG, Matthews SG, Gibb W, Lye SJ. Endocrine and paracrine regulation of birth at term and preterm. Endocr Rev 2000; 21:514-550.

99. Elf PK, Fivizzani AJ. Changes in sex steroid levels in yolks of the leghorn chicken, *Gallus domesticus*, during embryonic development. J Exp Zool 2002; 293:594-600.

100. Penning TM. Molecular endocrinology of hydroxysteroid dehydrogenases. Endocr Rev 1997; 18:281-305.

101. Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone-receptor forms A and B. EMBO J 1990; 9:1603-1614.

102. Tora L, Gronemeyer H, Turcotte B, Gaub MP, Chambon P. The N-terminal region of the chicken progesterone-receptor specifies target gene activation. Nature 1988; 333:185-188.

103. Graham JD, Clarke CL. Physiological action of progesterone in target tissues. Endocr Rev 1997; 18:502-519.

104. Goldman S, Weiss A, Almalah I, Shalev E. Progesterone receptor expression in human decidua and fetal membranes before and after contractions: possible mechanism for functional progesterone withdrawal. Mol Hum Reprod 2005; 11:269-277.

105. Pasanen S, Ylikomi T, Syvälä H, Tuohimaa P. Distribution of progesterone receptor in chicken: novel target organs for progesterone and estrogen action. Mol Cell Endocrinol 1997; 135:79-91.

106. Griffin C, Flouriot G, Sonntag-Buck V, Gannon F. Two functionally different protein isoforms are produced from the chicken estrogen receptor-alpha gene. Mol Endocrinol 1999; 13:1571-1587.

107. Walter LM, Rogers PAW, Girling JE. The role of progesterone in endometrial angiogenesis in pregnant and ovariectomised mice. Reproduction 2005; 129:765-777.

108. Girling JE, Lederman FL, Walter LM, Rogers PAW. Progesterone, but not estrogen, stimulates vessel maturation in the mouse endometrium. Endocrinology 2007; 148:5433-5441.

109. Cudeville C, Mondon F, Robert B, Rebourcet R, Mignot T-M, Benassayag C, Ferre F. Evidence for progesterone receptors in the human fetoplacental vascular tree. Biol Reprod 2000; 62:759-765.

110. Omar HA, Ramirez R, Gibson M. Properties of a progesterone-induced relaxation in human placental arteries and veins. J Clin Endocrinol Metab 1995; 80:370-373.

111. Strauss JF, Martinez F, Kiriakidou M. Placental steroid hormone synthesis: unique features and unanswered questions. Biol Reprod 1996; 54:303-311.

112. Conley AJ, Mason JI. Placental steroid hormones. Baillieres Clin Endocrinol Metab 1990; 4:249-272.

113. Evain-Brion D, Malassine A. Human placenta as an endocrine organ. Growth Horm IGF Res 2003; 13:S34-S37.

114. Jonathan R S. Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. Mol Cell Endocrinol 2001; 185:61-71.

115. Jansson T, Powell TL. Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. Clin Sci 2007; 113:1-13.

116. Albergotti LC, Hamlin HJ, McCoy MW, Guillette LJ, Jr. Endocrine activity of extraembryonic membranes extends beyond placental amniotes. PLoS ONE 2009; 4:e5452.

117. Shanker YG, Jagannadha Rao A. Progesterone receptor expression in the human placenta. Mol Hum Reprod 1999; 5:481-486.

118. Hsu TY, Lan KC, Tsai CC, Ou CY, Cheng BH, Tsai MY, Kang HY, Tung YH, Wong YH, Huang KE. Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies. Taiwan J Obstet Gyne 2009; 48:262-267.

119. Klemcke HG, Sampath Kumar R, Yang K, Vallet JL, Christenson RK. 11 beta-hydroxysteroid dehydrogenase and glucocorticoid receptor messenger RNA expression in porcine placentae: effects of stage of gestation, breed, and uterine environment. Biol Reprod 2003; 69:1945-1950.

120. Bukovsky A, Cekanova M, Caudle MR, Wimalasena J, Foster JS, Henley DC, Elder RF. Expression and localization of estrogen receptor-alpha protein in normal and abnormal term placentae and stimulation of trophoblast differentiation by estradiol. Reprod Biol Endocrinol 2003; 1:1-18.

121. Bukovsky A, Caudle MR, Cekanova M, Fernando RI, Wimalasena J, Foster JS, Henley DC, Elder RF. Placental expression of estrogen receptor beta and its hormone binding variant – comparison with estrogen receptor alpha and a role for estrogen receptors in asymmetric division and differentiation of estrogen-dependent cells. Reprod Biol Endocrinol 2003; 1:1-21.

122. Schimmer BP, White PC. Minireview: steroidogenic factor 1: its roles in differentiation, development, and disease. Mol Endocrinol 2010; 24:1322-1337.

123. Beato M, Klug J. Steroid hormone receptors: an update. Hum Reprod Update 2000; 6:225-236.

124. Lang JW, Andrews HV. Temperature-dependent sex determination in crocodilians. J Exp Zool 1994; 270:28-44.

125. Milnes MR, Jr, Roberts RN, Guillette LJ, Jr. Effects of incubation temperature and estrogen exposure on aromatase activity in the brain and gonads of embryonic alligators. Environ Health Perspect 2002; 110:393–396.

126. Durkee TJ, McLean MP, Hales DB, Payne AH, Waterman MR, Khan I, Gibori G. P450(17 alpha) and P450SCC gene expression and regulation in the rat placenta. Endocrinology 1992; 130:1309-1317.

127. Arensburg J, Payne AH, Orly J. Expression of steroidogenic genes in maternal and extraembryonic cells during early pregnancy in mice. Endocrinology 1999; 140:5220-5232.

128. Sood R, Zehnder JL, Druzin ML, Brown PO. Gene expression patterns in human placenta. Proc Natl Acad Sci USA 2006; 103:5478-5483.

129. Ferguson M. Reproductive biology and embryology of the crocodilians. In: Gans C, Maderson P, Billet F (eds.), Biology of the reptilia, vol. 14. New York: J. Wiley and Sons; 1985: 329–491.

130. Gunderson MP, Kohno S, Blumberg B, Iguchi T, Guillette LJ, Jr. Up-regulation of the alligator CYP3A77 gene by toxaphene and dexamethasone and its short term effect on plasma testosterone concentrations. Aquat Toxicol 2006; 78:272-283.

131. Kohno S, Bermudez DS, Katsu Y, Iguchi T, Guillette LJ, Jr. Gene expression patterns in juvenile American alligators (*Alligator mississippiensis*) exposed to environmental contaminants. Aquat Toxicol 2008; 88:95-101.

132. Milnes MR, Bryan TA, Katsu Y, Kohno S, Moore BC, Iguchi T, Guillette LJ, Jr. Increased posthatching mortality and loss of sexually dimorphic gene expression in alligators (*Alligator mississippiensis*) from a contaminated environment. Biol Reprod 2008; 78:932-938.

133. Moore BC, Milnes MR, Kohno S, Katsu Y, Iguchi T, Guillette LJ, Jr. Influences of sex, incubation temperature, and environmental quality on gonadal estrogen and androgen receptor messenger RNA expression in juvenile American alligators (*Alligator mississippiensis*). Biol Reprod 2010; 82:194-201.

134. Katsu Y, Bermudez DS, Braun EL, Helbing C, Miyagawa S, Gunderson MP, Kohno S, Bryan TA, Guillette LJ, Jr., Iguchi T. Molecular cloning of the estrogen and progesterone receptors of the American alligator. Gen Comp Endocrinol 2004; 136:122-133.

135. Conley AJ, Head JR, Stirling DT, Mason JI. Expression of steroidogenic enzymes in the bovine placenta and fetal adrenal glands throughout gestation. Endocrinology 1992; 130:2641-2650.

136. Lea RG, Wooding P, Stewart I, Hannah LT, Morton S, Wallace K, Aitken RP, Milne JS, Regnault TR, Anthony RV, Wallace JM. The expression of ovine placental lactogen, StAR and progesterone-associated steroidogenic enzymes in placentae of overnourished growing adolescent ewes. Reproduction 2007; 133:785-796.

137. Tuckey RC. Progesterone synthesis by the human placenta. Placenta 2005; 26:273-281.

138. Johnson DC. Cellular localization and factors controlling rat placental cytochrome P45017 alpha (CYP17): 17 alpha-hydroxylase/C17,20-lyase activity. Biol Reprod 1992; 46:30-38.

139. Mason JI, France JT, Magness RR, Murry BA, Rosenfeld CR. Ovine placental steroid 17{alpha}-hydroxylase/C-17,20-lyase, aromatase and sulphatase in dexamethasone-induced and natural parturition. J Endocrinol 1989; 122:351-359.

140. Albrecht ED, Pepe GJ. Placental steroid hormone biosynthesis in primate pregnancy. Endocr Rev 1990; 11:124-150.

141. Kamat A, Graves KH, Smith ME, Richardson JA, Mendelson CR. A 500-bp region, 40 kb upstream of the human CYP19 (aromatase) gene, mediates placenta-specific expression in transgenic mice. Proc Natl Acad Sci USA 1999; 96:4575-4580.

142. Kamat A, Hinshelwood MM, Murry BA, Mendelson CR. Mechanisms in tissue-specific regulation of estrogen biosynthesis in humans. Trends Endocrin Met 2002; 13:122-128.

143. Hahn T, Barth S, Graf R, Engelmann M, Beslagic D, Reul JMHM, Holsboer F, Dohr G, Desoye G. Placental glucose transporter expression is regulated by glucocorticoids. J Clin Endocrinol Metab 1999; 84:1445-1452.

144. Robinson BG, Emanuel RL, Frim DM, Majzoub JA. Glucocorticoid stimulates expression of corticotropin-releasing hormone gene in human placenta. P Nat Acad Sci 1988; 85:5244-5248.

145. Baisden B, Sonne S, Joshi RM, Ganapathy V, Shekhawat PS. Antenatal dexamethasone treatment leads to changes in gene expression in a murine late placenta. Placenta 2007; 28:1082-1090.

146. Hewitt DP, Mark PJ, Waddell BJ. Glucocorticoids prevent the normal increase in placental vascular endothelial growth factor expression and placental vascularity during late pregnancy in the rat. Endocrinology 2006; 147:5568-5574.

147. Whittle WL, Patel FA, Alfaidy N, Holloway AC, Fraser M, Gyomorey S, Lye SJ, Gibb W, Challis JRG. Glucocorticoid regulation of human and ovine parturition: the relationship between fetal hypothalamic-pituitary-adrenal axis activation and intrauterine prostaglandin production. Biol Reprod 2001; 64:1019-1032.

148. Shanker YG, Rao AJ. Regulation of progresterone biosynthesis in the human placenta by estradiol 17β and progesterone. IUBMB Life 1997; 43:591-599.

149. Ferguson MWJ. Post-laying stages of embryonic development for crocodilians. In: Webb G, Manolis JW, Charlie S, Whitehead PJ (eds.), Wildlife management: crocodiles and alligators. Chipping Norton, Australia: Surrey Beatty & Sons Pty Limited; 1987.

150. Medler KF, Lance VA. Sex differences in plasma corticosterone levels in alligator (*Alligator mississippiensis*) embryos. J Exp Zool 1998; 280:238-244.

151. Kalliecharan R, Hall BK. A developmental study of the levels of progesterone, corticosterone, cortisol, and cortisone circulating in plasma of chick embryos. Gen Comp Endocrinol 1974; 24:364-372.

152. Clifton VL. Review: sex and the human placenta: mediating differential strategies of fetal growth and survival. Placenta; 31:S33-S39.

153. Mao J, Zhang X, Sieli PT, Falduto MT, Torres KE, Rosenfeld CS. Contrasting effects of different maternal diets on sexually dimorphic gene expression in the murine placenta. P Nat Acad Sci 2010; 107:5557-5562.

154. Bergeron JM, Gahr M, Horan K, Wibbels T, Crews D. Cloning and in situ hybridization analysis of estrogen receptor in the developing gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. Dev Growth & Differ 1998; 40:243-254.

155. Elf PK. Yolk steroid hormones and sex determination in reptiles with TSD. Gen Comp Endocrinol 2003; 132:349-355.

156. Pieau C, Dorizzi M. Oestrogens and temperature-dependent sex determination in reptiles: all is in the gonads. J Endocrinol 2004; 181:367-377.

157. Ramsey M, Crews D. Steroid signaling and temperature-dependent sex determination - reviewing the evidence for early action of estrogen during ovarian determination in turtles. Semin Cell Dev Biol 2009; 20:283-292.

158. Clark BJ, Stocco DM. StAR-A tissue specific acute mediator of steroidogenesis. Trends Endocrin Met 1996; 7:227-233.

159. Groothuis TGG, Schwabl H. Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? Philos T R Soc B 2008; 363:1647-1661.

160. Hamlin HJ, Lowers RH, Albergotti LC, McCoy MW, Mutz J, Guillette LJ, Jr. . Environmental influence on yolk steroids in American alligators (*Alligator mississippiensis*). Biol Reprod 2010; 83:736–741.

161. Bowden RM, Ewert MA, Nelson CE. Hormone levels in yolk decline throughout development in the red-eared slider turtle (*Trachemys scripta elegans*). Gen Comp Endocrinol 2002; 129:171-177.

162. Paitz RT, Bowden RM. Rapid decline in the concentrations of three yolk steroids during development: Is it embryonic regulation? Gen Comp Endocrinol 2009; 161:246-251.

163. Conley AJ, Elf P, Corbin CJ, Dubowsky S, Fivizzani A, Lang JW. Yolk steroids decline during sexual differentiation in the alligator. Gen Comp Endocrinol 1997; 107:191-200.

164. Woods JE, Podczaski ES. Androgen synthesis in the gonads of the chick embryo. Gen Comp Endocrinol 1974; 24:413-423.

165. Woods JE, Simpson RM, Moore PL. Plasma testosterone levels in the chick embryo. Gen Comp Endocrinol 1975; 27:543-547.

166. Gilbert L, Bulmer E, Arnold KE, Graves JA. Yolk androgens and embryo sex: maternal effects or confounding factors? Horm Behav 2007; 51:231-238.

167. Paitz RT, Bowden RM, Casto JM. Embryonic modulation of maternal steroids in European starlings (*Sturnus vulgaris*). P R Soc B 2010; 278:99-106.

168. Tong MH, Jiang H, Liu P, Lawson JA, Brass LF, Song W-C. Spontaneous fetal loss caused by placental thrombosis in estrogen sulfotransferase deficient mice. Nat Med 2005; 11:153-159.

169. Reed MJ, Purohit A, Woo LWL, Newman SP, Potter BVL. Steroid sulfatase: molecular biology, regulation, and inhibition. Endocr Rev 2005; 26:171-202.

170. Paitz RT, Bowden RM. A proposed role of the sulfotransferase/sulfatase pathway in modulating yolk steroid effects. Integr Comp Biol 2008; 48:419-427.

171. Romanoff AL. The avian embryo. New York Macmillan; 1960.

172. Mess A, Carter AM. Evolution of the placenta during the early radiation of placental mammals. Comp Biochem Phys A 2007; 148:769-779.

173. Stewart ME, Terepka AR. Transport functions of the chick chorio-allantoic membrane: normal histology and evidence for active electrolyte transport from the allantoic fluid, in vivo. Exp Cell Res 1969; 58:93-106.

174. Munro HN, Pilistine SJ, Fant ME. The Placenta in nutrition. Annu Rev Nutr 1983; 3:97-124.

175. Blackburn DG. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. Am Zool 1992; 32:313-321.

176. Levitz M. Conjugation and transfer of fetal-placental steroid hormones. J Clin Endocrinol Metab 1966; 26:773-777.

177. Lee MJ, Wang Z, Yee H, Ma Y, Swenson N, Yang L, Kadner SS, Baergen RN, Logan SK, Garabedian MJ, Guller S. Expression and regulation of glucocorticoid receptor in human placental villous fibroblasts. Endocrinology 2005; 146:4619-4626.

178. Ogle TF, Mills TM, Soares MJ. Changes in cytosolic and nuclear progesterone receptors during pregnancy in rat placenta. Biol Reprod 1989; 40:1012-1019.

179. Pieber D, Allport VC, Hills F, Johnson M, Bennett PR. Interactions between progesterone receptor isoforms in myometrial cells in human labour. Mol Hum Reprod 2001; 7:875-879.

180. Zardoya R, Meyer A. The evolutionary position of turtles revised. Naturwissenschaften 2001; 88:193-200.

181. Blackburn DG. Viviparity and oviparity: evolution and reproductive strategies. In: Knobil TE, Neill JD (eds.), Encyclopedia of reproduction, vol. 4. London, UK: Academic Press; 1999: 994-1003.

182. Enge KM, Percival HF, Rice KG, Jennings ML, Masson GR, Woodward AR. Summer nesting of turtles in alligator nests in Florida. J Herpetol 2000; 34:497-503.

183. Yntema CL. A series of stages in the embryonic development of *Chelydra serpentina*. J Morphol 1968; 125:219-251.

184. Ewert MA, Etchberger CR, Nelson CE. Turtle sex-determining modes and TSD patterns, and some TSD patterns correlates. In: Valenzuela N, Bull JJ (eds.), Temperature-dependent sex determination: Smithsonian Books; 2004.

185. Neill WT. Viviparity in snakes: Some ecological and zoogeographical considerations. Am Nat 1964; 98:35-55.

186. Ogle TF. Characteristics of high affinity progesterone binding to rat placental cytosol. Endocrinology 1980; 106:1861-1868.

187. Reynolds LP, Redmer DA. Angiogenesis in the placenta. Biol Reprod 2001; 64:1033-1040.

188. Wilting J, Christ B, Bokeloh M, Weich HA. In vivo effects of vascular endothelial growth factor on the chicken chorioallantoic membrane. Cell Tissue Res 1993; 274:163-172.

189. Ribatti D, Nico B, Vacca A, Roncali L, Burri PH, Djonov V. Chorioallantoic membrane capillary bed: A useful target for studying angiogenesis and anti-angiogenesis in vivo. Anat Rec 2001; 264:317-324.

190. Ancelin M, Buteau-Lozano H, Meduri G, Osborne-Pellegrin M, Sordello S, Plouët J, Perrot-Applanat M. A dynamic shift of VEGF isoforms with a transient and selective progesterone-induced expression of VEGF189 regulates angiogenesis and vascular permeability in human uterus. Proc Natl Acad Sci USA 2002; 99:6023-6028.

191. Rider V, Carlone DL, Foster RT. Oestrogen and progesterone control basic fibroblast growth factor mRNA in the rat uterus. J Endocrinol 1997; 154:75-84.

192. Tsai MJ, BW OM. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem 1994:451-486.

193. Fujimoto J, Nakagawa Y, Toyoki H, Sakaguchi H, Sato E, Tamaya T. Estrogen-related receptor expression in placenta throughout gestation. J Steroid Biochem Mol Biol 2005; 94:67-69.

194. Pikulkaew S, De Nadai A, Belvedere P, Colombo L, Dalla Valle L. Expression analysis of steroid hormone receptor mRNAs during zebrafish embryogenesis. Gen Comp Endocrinol 2010; 165:215-220.

195. Speake BK, Murray AMB, Noble RC. Transport and transformations of yolk lipids during development of the avian embryo. Prog Lipid Res 1998; 37:1-32.

196. Noble RC, Connor K, Smith WK. The synthesis and accumulation of cholesterol esters by the developing embryo of domestic fowl. Poult Sci 1984; 63:558-564.

197. Azuma M, Seki T, Fujishita S. Changes of egg retinoids during the development of *Xenopus laevis*. Vision Res 1990; 30:1395-1400.

198. Greenblatt M, Brown CL, Lee M, Dauder S, Bern HA. Changes in thyroid hormone levels in eggs and larvae and in iodide uptake by eggs of coho and chinook salmon, *Oncorhynchus kisutsch* and *O. tschawytscha*. Fish Phys Biochem 2005; 6:261-278.

199. Hwang P-P, Wu S-M, Lin J-H, Wu L-S. Cortisol content of eggs and larvae of teleosts. Gen Comp Endocrinol 1992; 86:189-196.

200. Irie T, Seki T. Retinoid composition and retinal localization in the eggs of teleost fishes. Comp Biochem Physiol B 2002; 131:209-219.

201. Kobuke L, Specker JL, Bern HA. Thyroxine content of eggs and larvae of coho salmon, *Oncorhynchus kisutch*. J Exp Zool 1987; 242:89-94.

202. Lam TJ. Hormones and egg/larval quality in fish. J World Aquac Soc 1994; 25:2-12.

203. Weber GM, Farrar ES, Tom CKF, Grau EG. Changes in whole-body thyroxine and triiodothyronine concentrations and total content during early development and metamorphosis of the toad *Bufo marinus*. Gen Comp Endocrinol 1994; 94:62-71.

204. Hanken J, Jennings DH, Olsson L. Mechanistic basis of life-history evolution in anuran amphibians: direct development. Amer Zool 1997; 37:160-171.

205. Ezaz T, Stiglec R, Veyrunes F, Marshall Graves JA. Relationships between vertebrate ZW and XY sex chromosome systems. Curr Biol 2006; 16:R736-R743.

206. Cobb GP, Bargar TA, Pepper CB, Norman DM, Houlis PD, Anderson TA. Using chorioallantoic membranes for non-lethal assessment of persistent organic pollutant exposure and effect in oviparous wildlife. Ecotoxicology 2003; 12:31-45.

207. Peakall DB. DDE: its presence in peregrine eggs in 1948. Science 1974; 183:673-674.

208. Annas A, Granberg L, Strandberg W, Brandt I, Brittebo EB, Brunström B. Basal and induced EROD activity in the chorioallantoic membrane during chicken embryo development. Environ Toxicol Phar 1999; 8:49-52.

209. Granberg L, Brunström B, Brandt I. Formation of benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene adducts in vascular endothelia of cytochrome P4501A–induced chicken embryos. Environ Toxicol Chem 2003; 22:2393-2399.

210. Peakal DB, Lew TS, Springer AM, Walker Ip W, Risebrough RW, Monk JG, Jarman WM, Walton BJ, Reynolds LM, Fyfe RW, Kiff LF. Determination of the DDE and PCB contents of peregrine falcon eggs: A comparison of whole egg measurements and estimates derived from eggshell membranes. Arch Environ Contam Toxicol 1983; 12:523-528.

211. Cobb GP, Norman DM, Kendall RJ. Organochlorine contaminant assessment in great blue herons using traditional and nonlethal monitoring techniques. Environ Pollut 1994; 83:299-309.

212. Cobb GP, Wood PD, O'Quinn M. Polychlorinated biphenyls in eggs and chorioallantoic membranes of American alligators (*Alligator mississippiensis*) from coastal South Carolina. Environ Toxicol Chem 1997; 16:1456-1462.

213. Colborn T, Clement C (eds.). Chemically-induced alterations in sexual and functional development: The wildlife/human connection. Princeton: Princeton University Press; 1992.

214. Guillette LJ, Jr. Endocrine disrupting contaminants-beyond the dogma. Environ Health Perspect 2005; 114.

215. Fisher JS. Are all EDC effects mediated via steroid hormone receptors? Toxicology 2004; 205:33-41.

216. Crain DA, Eriksen M, Iguchi T, Jobling S, Laufer H, LeBlanc GA, Guillette LJ, Jr. An ecological assessment of bisphenol-A: evidence from comparative biology. Reprod Toxicol 2007; 24:225-239.

217. Dodds EC, Lawson W. Synthetic oestrogenic agents without the phenanthrene nucleus. Nature (Lond) 1936; 137.

218. Vandenberg LN, Maffini MV, Sonnenschein C, Rubin BS, Soto AM. Bisphenol-A and the great divide: a review of controversies in the field of endocrine disruption. Endocr Rev 2009; 30:75-95.

219. Vom Saal FS, Hughes C. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. Environ Health Perspect 2005; 113:926-933.

220. Rubin BS, Murray MK, Damassa DA, King JC, Soto AM. Perinatal exposure to low doses of bisphenol A affects body weight, patterns of estrous cyclicity, and plasma LH levels. Environ Health Perspect 2001; 109.

221. Council AC. http://www.bisphenol-a.org/index.html. In. Washington, DC; 2011.

222. Annas A, Brunström B, Brittebo EB. CYP1A-dependent activation of xenobiotics in endothelial linings of the chorioallantoic membrane (CAM) in birds. Arch Toxicol 2000; 74:335-342.

223. Agency USEP. A cross-species mode of action information assessment: a case study of bisphenol A. In: Assessment NCfE (ed.). Washington, DC; 2005.

224. Levy G, Lutz I, Krüger A, Kloas W. Bisphenol A induces feminization in *Xenopus laevis* tadpoles. Environ Res 2004; 94:102-111.

225. Laws SC, Carey SA, Ferrell JM, Bodman GJ, Cooper RL. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. Toxicol Sci 2000; 54:154-167.

226. Ing NH, Tornesi MB. Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. Biol Reprod 1997; 56:1205-1215.

227. Bergeron RM, Thompson TB, Leonard LS, Pluta L, Gaido KW. Estrogenicity of bisphenol A in a human endometrial carcinoma cell line. Mol Cell Endocrinol 1999; 150:179-187.

228. Kumar P, Kamat A, Mendelson CR. Estrogen receptor α (ERα) mediates stimulatory effects of estrogen on aromatase (CYP19) gene expression in human placenta. Mol Endocrinol 2009; 23:784-793.

229. Kinoshita Y, Chen S. Induction of aromatase (CYP19) expression in breast cancer cells through a nongenomic action of estrogen receptor α. Cancer Res 2003; 63:3546-3555.

230. Tsai CL, Wang LH, Fang LS. Estradiol and *para*-chlorophenylalanine downregulate the expression of brain aromatase and estrogen receptor α mRNA during the critical period of feminization in tilapia (*Oreochromis mossambicus*). Neuroendocrinology 2001; 74:325-334.

231. Zhou W, Liu J, Liao L, Han S, Liu J. Effect of bisphenol A on steroid hormone production in rat ovarian theca-interstitial and granulosa cells. Mol Cell Endocrinol 2008; 283:12-18.

232. Huang H, Leung LK. Bisphenol A downregulates CYP19 transcription in JEG-3 cells. Toxicol Lett 2009; 189:248-252.

233. Micevych P, Soma KK, Sinchak K. Neuroprogesterone: key to estrogen positive feedback? Brain Res Rev 2008; 57:470-480.

234. Beaudoin C, Blomquist C, Bonenfant M, Tremblay Y. Expression of the genes for 3 beta-hydroxysteroid dehydrogenase type 1 and cytochrome P450scc during syncytium formation by human placental cytotrophoblast cells in culture and the regulation by progesterone and estradiol. J Endocrinol 1997; 154:379-387.

235. Govoroun M, McMeel OM, Mecherouki H, Smith TJ, Guiguen Y. 17β-estradiol treatment decreases steroidogenic enzyme messenger ribonucleic acid levels in the rainbow trout testis. Endocrinology 2001; 142:1841-1848.

236. Freeman DA. Estradiol acts as a competitive inhibitor of the 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase enzyme of cultured leydig tumor cells. Endocrinology 1985; 117:2127-2133.

237. Ye L, Zhao B, Hu G, Chu Y, Ge R-S. Inhibition of human and rat testicular steroidogenic enzyme activities by bisphenol A. Toxicol Lett 2011; 207:137-142.

238. Hardin B, Bond G, Sikov M, Andrew F, Beliles R, Niemeier R. Testing of selected workplace chemicals for teratogenic potential. Scand J Work Environ Health 1981; 7:66-75.

239. Ramakrishnan S, Wayne NL. Impact of bisphenol-A on early embryonic development and reproductive maturation. Reprod Toxicol 2008; 25:177-183.

240. Savabieasfahani M, Kannan K, Astapova O, Evans NP, Padmanabhan V. Developmental programming: differential effects of prenatal exposure to bisphenol-A or methoxychlor on reproductive function. Endocrinology 2006; 147:5956-5966.

241. Zimmerman SA, Clevenger WR, Brimhall BB, Bradshaw WS. Diethylstilbestrol-induced perinatal lethality in the rat. II. Perturbation of parturition. Biol Reprod 1991; 44:583-589.

242. Stoker C, Beldomenico PM, Bosquiazzo VL, Zayas MA, Rey F, Rodriguez H, Munoz-de-Toro M, Luque EH. Developmental exposure to endocrine disruptor chemicals alters follicular dynamics and steroid levels in *Caiman latirostris*. Gen Comp Endocrinol 2008; 156:603-612.

243. Stoker C, Rey F, Rodriguez H, Ramos JG, Sirosky P, Larriera A, Luque EH, Munoz-de-Toro M. Sex reversal effects on *Caiman latirostris* exposed to environmentally relevant doses of the xenoestrogen bisphenol A. Gen Comp Endocrinol 2003; 133:287-296.

244. Hall JM, Couse JF, Korach KS. The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 2001; 276:36869-36872.

245. Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J-Å, Nilsson S. Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists. Mol Pharmacol 1998; 54:105-112.

246. Hall JM, McDonnell DP. The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 1999; 140:5566-5578.

247. Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Häggblad J, Nilsson S, Gustafsson J-Å. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997; 138:863-870.

248. Sakimura M, Tsukada A, Usami M, Hanzawa S, Saito N, Ohno Y, Shimada K. Effect of estradiol and nonylphenol on mRNA expression of estrogen receptors α and β, and cytochrome P450 aromatase in the gonad of chicken embryos. J Poult Sci 2002; 39:302-309.

249. Sabo-Attwood T, Blum JL, Kroll KJ, Patel V, Birkholz D, Szabo NJ, Fisher SZ, McKenna R, Campbell-Thompson M, Denslow ND. Distinct expression and activity profiles of largemouth bass (*Micropterus salmoides*) estrogen receptors in response to estradiol and nonylphenol. J Mol Endocrinol 2007; 39:223-237.

250. Crews D, Bull JJ, Wibbels T. Estrogen and sex reversal in turtles: a dose-dependent phenomenon. Gen Comp Endocrinol 1991; 81:357-364.

251. Podreka S, Georges A, Maher B, Limpus CJ. The environmental contaminant DDE fails to influence the outcome of sexual differentiation in the marine turtle *Chelonia mydas*. Environ Health Perspect 1998; 106.

252. Behrman RE, Butler AS (eds.). Preterm birth: causes, consequences, and prevention. The National Academies Press; 2007.

253. Stillerman KP, Mattison DR, Giudice LC, Woodruff TJ. Environmental exposures and adverse pregnancy outcomes: a review of the science. Reprod Sci 2008; 15:631-650.

254. Martin JA, Hamilton BE, Sutton PD, Ventura SJ, Menacker F, Kirmeyer S, Mathews TJ. Births: Final data for 2006. National vital statistics reports. In, vol. 57. Hyattsville, MD: National Center for Health Statistics; 2009.

255. Davidoff MJ, Dias T, Damus K, Russell R, Bettegowda VR, Dolan S, Schwarz RH, Green NS, Petrini J. Changes in the gestational age distribution among U.S. singleton births: impact on rates of late preterm birth, 1992 to 2002. Semin Perinatol 2006; 30:8-15.

256. Brackbill Y, Berendes HW. Dangers of diethylstilboestrol: review of a 1953 paper. Lancet 1978; 2:520.

257. Hatch EE, Troisi R, Wise LA, Titus-Ernstoff L, Hyer M, Palmer JR, Strohsnitter WC, Robboy SJ, Anderson D, Kaufman R, Adam E, Hoover RN. Preterm birth, fetal growth, and age at menarche among women exposed prenatally to diethylstilbestrol (DES). Reprod Toxicol 2011; 31:151-157.

258. Henson MC, Chedrese PJ. Endocrine disruption by cadmium, a common environmental toxicant with paradoxical effects on reproduction. Exp Biol Med 2004; 229:383-392.

259. Longnecker MP, Klebanoff MA, Zhou H, Brock JW. Association between maternal serum concentration of the DDT metabolite DDE and preterm and small-for-gestational-age babies at birth. The Lancet 2001; 358:110-114.

260. Pathak R, Ahmed RS, Tripathi AK, Guleria K, Sharma CS, Makhijani SD, Banerjee BD. Maternal and cord blood levels of organochlorine pesticides: association with preterm labor. Clin Biochem 2009; 42:746-749.

261. DeLong RL, Gilmartin WG, Simpson JG. Premature births in California Sea Lions: association with high organochlorine pollutant residue levels. Science 1973; 181:1168-1170.

262. Cantonwine D, Meeker J, Hu H, Sanchez B, Lamadrid-Figueroa H, Mercado-Garcia A, Fortenberry G, Calafat A, Tellez-Rojo M. Bisphenol A exposure in Mexico City and risk of prematurity: a pilot nested case control study. Environ Health 2010; 9:62.

263. Ranjit N, Siefert K, Padmanabhan V. Bisphenol-A and disparities in birth outcomes: a review and directions for future research. J Perinatol 2010; 30:2-9.

264. Furusawa N. Transferring and distributing profiles of p,p′-(DDT) in egg-forming tissues and eggs of laying hens following a single oral administration. J Vet Med A 2002; 49:334-336.

265. Terepka AR, Stewart ME, Merkel N. Transport functions of the chick chorio-allantoic membrane: II. Active calcium transport, in vitro. Exp Cell Res 1969; 58:107-117.

266. Crain DA, Guillette LJ, Jr, Rooney AA, Pickford DB. Alterations in steroidogenesis in alligators (*Alligator mississippiensis*) exposed naturally and experimentally to environmental contaminants. Environ Health Perspect 1997; 105:528–533.

267. Bergeron JM, Crews D, McLachlan JA. PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. Environ Health Perspect 1994; 102:780–781.

268. Solla SRd, Martin PA. Absorption of current use pesticides by snapping turtle (*Chelydra serpentina*) eggs in treated soil. Chemosphere 2011.

269. Domoradzki JY, Pottenger LH, Thornton CM, Hansen SC, Card TL, Markham DA, Dryzga MD, Shiotsuka RN, Waechter JM. Metabolism and pharmacokinetics of bisphenol A (BPA) and the embryo-fetal distribution of BPA and BPA-monoglucuronide in CD Sprague-Dawley rats at three gestational stages. Toxicol Sci 2003; 76:21-34.

270. Schönfelder G, Wittfoht W, Hopp H, Talsness CE, Paul M, Chahoud I. Parent bisphenol A accumulation in the human maternal-fetal-placental unit. Environ Health Perspect 2002; 110:doi:10.1289/ehp.021100703.

271. Vandenberg LN, Hauser R, Marcus M, Olea N, Welshons WV. Human exposure to bisphenol A (BPA). Reprod Toxicol 2007; 24:139-177.

272. Halldin K, Berg C, Bergman Å, Brandt I, Brunström B. Distribution of bisphenol A and tetrabromobisphenol A in quail eggs, embryos and laying birds and studies on reproduction variables in adults following in ovo exposure. Arch Toxicol 2001; 75:597-603.

273. Takayanagi S, Tokunaga T, Liu X, Okada H, Matsushima A, Shimohigashi Y. Endocrine disruptor bisphenol A strongly binds to human estrogen-related receptor γ (ERRγ) with high constitutive activity. Toxicol Lett 2006; 167:95-105.

274. Takeda Y, Liu X, Sumiyoshi M, Matsushima A, Shimohigashi M, Shimohigashi Y. Placenta expressing the greatest quantity of bisphenol A receptor ERRγ among the human reproductive tissues: predominant expression of type-1 ERRγ isoform. J Biochem (Tokyo) 2009; 146:113-122.

275. Escobar JC, Patel SS, Beshay VE, Suzuki T, Carr BR. The human placenta expresses CYP17 and generates androgens de novo. J Clin Endocrinol Metab 2011; 96:1385-1392.

276. Mukherjee S, James JL, Thilaganathan B, Whitley GSJ, Michael AE, Cartwright JE. Elevated glucocorticoid metabolism in placental tissue from first trimester pregnancies at increased risk of pre-eclampsia. Placenta 2011; 32:687-693.

277. Sathishkumar K, Balakrishnan M, Chinnathambi V, Chauhan M, Hankins GDV, Yallampalli C. Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia. J Perinatol 2011.

278. Wake MH. Evolutionary scenarios, homology and convergence of structural specializations for vertebrate viviparity. Am Zool 1992; 32:256-263.

279. Schwarz BE, Milewich L, Gant NF, Johnston JM, MacDonald PC, Porter JC. Progesterone binding and metabolism in human fetal membranes. Ann N Y Acad Sci 1977; 286:304-312.

280. Stewart PM, Rogerson FM, Mason JI. Type 2 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid and activity in human placenta and fetal membranes: its relationship to birth weight and putative role in fetal adrenal steroidogenesis. J Clin Endocrinol Metab 1995; 80:885-890.

281. Hobkirk R. Steroid sulfotransferases and steroid sulfate sulfatases: characteristics and biological roles. Can J Biochem Cell Biol 1985; 63:1127-1144.

282. Taylor AH, McParland PC, Taylor DJ, Bell SC. The progesterone receptor in human term amniochorion and placenta is isoform C. Endocrinology 2006; 147:687-693.

283. Sun M, Ramirez M, Challis JRG, Gibb W. Immunohistochemical localization of the glucocorticoid receptor in human fetal membranes and decidua at term and preterm delivery. J Endocrinol 1996; 149:243-248.

284. Andrews RM. Patterns of embryonic development. In: Deeming DC (ed.) Reptilian incubation: environment, evolution and behaviour. Nottingham: Nottingham University Press; 2004: 75–102.

285. Andrews RM. Evolution of viviparity in squamate reptiles (*Sceloporus* spp.): a variant of the cold-climate model. J Zool 2000; 250:243-253.

286. Breit S, Ashman K, Wilting J, Rössler J, Hatzi E, Fotsis T, Schweigerer L. The N-myc oncogene in human neuroblastoma cells: down-regulation of an angiogenesis inhibitor identified as activin A. Cancer Res 2000; 60:4596-4601.

287. Struman I, Bentzien F, Lee H, Mainfroid V, D'Angelo G, Goffin V, Weiner RI, Martial JA. Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. Proc Nat Acad Sci USA 1999; 96:1246-1251.

288. Wei L-H, Kuo M-L, Chen C-A, Chou C-H, Lai K-B, Lee C-N, Hsieh C-Y. Interleukin-6 promotes cervical tumor growth by VEGF-dependent angiogenesis via a STAT3 pathway. Oncogene 2003; 22:1517-1527.

289. Form DM, Auerbach R. PGE2 and angiogenesis. Proc Soc Exp Biol Med 1983; 172:214-218.

BIOGRAPHICAL SKETCH

Lori Cruze Albergotti was born in Knoxville, Tennessee. She graduated from Farragut High School in 1990 and attended Pellissippi State Technical Community College before transferring to the University of Tennessee where she graduated *Cum Laude* in 1997 with a Bachelor's of Science in Zoology. During her undergraduate tenure, Lori worked as a research assistant in the laboratory of Neil Greenberg where she assisted with behavioral assays investigating the physiology of aggressive and reproductive behavior in the green anole (*Anolis carolinensis*). After graduation, Lori went on to work as a veterinary technician for a number of years in South Carolina, Alaska and Oregon before returning to science.

In 2004, Lori joined the laboratory of Patrick Phillips as a research technician in the Center for Ecology and Evolutionary Biology at the University of Oregon. In the Phillips lab, Lori investigated variation in thermal preference among natural isolates of the soil nematode, *Caenorhabditis elegans*. This study provided the first empirical test of the classical adaptive hypothesis, which predicts that organisms should prefer temperatures that maximize fitness. As a result of this work, Lori co-authored two publications and received a student presentation award at the 2006 Meeting of Evolutionary Biologists of the Pacific Northwest.

After two years at the University of Oregon, Lori moved to the University of Florida and began her PhD in the Department of Zoology with Dr. Louis Guillette, Jr. Lori has assisted in teaching one semester of Evolutionary Developmental Biology and one semester of Communicating Complexity in Science. During her graduate degree, Lori has been heavily involved with mentoring students in research activities and has

mentored nine undergraduate and one high school student, five of whom are from underrepresented groups. Lori served as a Howard Hughes Medical Institute G.A.T.O.R (Group Advantaged Training of Research) mentor from 2007-2008 and as assistant director from 2009-2010. Lori mentored two students who have presented their work at international conferences and five that have presented their work at University of Florida symposiums.

In terms of Departmental service, Lori served as a coordinator for the Undergraduate Research Assistantship Program from 2006-2008 and was the student representative on the Physiology job search committee in 2009. Lori served as president of the Biology Graduate Student Association and was the student representative on the advisory committee from 2009-2010. She also served as a representative on the Graduate Student Fundraising Committee from 2010-2011. Outside of the Biology Department, Lori has served as an Alachua County Science and Engineering Fair judge in 2008 and 2010. She also traveled to Tulane University in New Orleans to participate in an undergraduate mentoring workshop entitled “Graduate School and Beyond: Pathways to Careers in Biological Research” in 2010.

Lori was awarded an Alumni fellowship at the University of Florida in 2006 and a National Science Foundation Graduate Research Fellowship in 2008. She received a Trainee poster award at the International Gordon Research Conference on Environmental Endocrine Disruptors in 2008 and as a result gave an impromptu oral presentation at that meeting. In addition, Lori’s research has been funded by two Sigma Xi Grants-in-aid of Research awards, one of which was awarded to her undergraduate student. In 2010, Lori was an invited speaker at the International Environmental

Hormone Conference in New Orleans. She has co-authored one book chapter and three peer reviewed publications during her time at the University of Florida, one of which received the Best Graduate Student Paper award for the Department of Biology in 2009.