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Comparative genomics of hormonal signaling in the chorioallantoic membrane of oviparous and viviparous amniotes

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ABSTRACT

In oviparous amniotes (reptiles, birds, and mammals) the chorioallantoic membrane (CAM) lines the inside of the egg and acts as the living point of contact between the embryo and the outside world. In livebearing (viviparous) amniotes, communication during embryonic development occurs across placental tissues, which form between the uterine tissue of the mother and the CAM of the embryo. In both oviparous and viviparous taxa, the CAM is at the interface of the embryo and the external environment and can transfer signals from there to the embryo proper. To understand the evolution of placental hormone production in amniotes, we examined the expression of genes involved in hormone synthesis, metabolism, and hormone receptivity in the CAM of species across the amniote phylogeny. We collected transcriptome data for the chorioallantoic membranes of the chicken (oviparous), the lizards *Lerista bougainvillii* (both oviparous and viviparous populations) and *Pseudemoia entrecasteauxii* (viviparous), and the horse *Equus caballus* (viviparous). The viviparous taxa differ in their mechanisms of nutrient provisioning: *L. bougainvillii* is lecithotrophic (embryonic nourishment is provided via the yolk only), but *P. entrecasteauxii* and the horse are placentotrophic (embryos are nourished via placental transport). Of the 423 hormone-related genes that we examined, 91 genes are expressed in all studied species, suggesting that the chorioallantoic membrane ancestrally had an endocrine function. Therefore, the chorioallantoic membrane appears to be a highly hormonally active organ in all amniotes. No genes are expressed only in viviparous species, suggesting that the evolution of viviparity has not required the recruitment of any specific hormone-related genes. Our data suggest that the endocrine function of the CAM as a placental tissue evolved in part through co-option of ancestral gene expression patterns.

1. Introduction

Reproduction is fundamental for organisms because it is the only mechanism that allows genes to be transmitted through generations. The ancestral reproductive mode for amniote vertebrates (reptiles, birds, and mammals) is egg-laying (oviparity), but live birth (viviparity) has evolved independently multiple times in reptiles, and once in mammals (Blackburn, 2014; Griffith et al., 2015). Embryonic development requires a suitable supply of respiratory gases and water. In viviparous species where the embryo is not exposed to the external environment, adequate structures are required to facilitate the exchange of materials between mother and offspring. In oviparous species, the chorioallantoic membrane (CAM, Fig. 1) is an embryonic tissue that lines the majority of the internal surface of the eggshell late in incubation, where it is the primary gas exchange organ between the embryo and the external environment (Piiper et al., 1980).

In contrast, in viviparous amniotes transfer of materials between mother and offspring occurs in a placenta composed of both maternal (uterine) and embryonic (chorioallantoic or yolk sac membranes) tissues (Blackburn, 2006; Van Dyke et al., 2014a; Wake, 1992). In eutherian mammals, the definitive placenta is produced by the uterus and the embryonic CAM. Chorioallantoic placentae are also found in all viviparous squamate reptiles, with the placental region in some species having morphological and physiological specializations for gas exchange and nutrient

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transport (Adams et al., 2005; Biazik et al., 2010; Blackburn, 1993; Griffith et al., 2013a; Van Dyke et al., 2015). Viviparous amniote placentae are extremely diverse in structure and function, with some placentae transporting respiratory gases, water, and small amounts of other molecules, and others transporting all classes of nutrients required for embryonic development (placentotrophy) (Ferner and Mess, 2011; Thompson and Speake, 2002; Thompson et al., 2002). Placentotrophy has evolved relatively few times in amniotes, and exists along a continuum, with complete reliance on egg yolk resources (lecithotrophy, in most squamates) at one end and complete reliance on placental nutrient transfer on the other end (as in eutherian mammals and some lizards). There are also species with intermediate reliance on both placental transfer and ovulated yolk resources, such as the lizard *Pseudemoia entrecasteauxii* (Ferner and Mess, 2011; Thompson et al., 2000; Van Dyke et al., 2014b).

To understand the evolution of viviparity, it is essential to understand the processes that regulate key pregnancy functions and how these processes have been modified during evolutionary transitions (Thompson and Speake, 2006). Hormones play an essential role in almost every step of amniote reproduction. Hormonal signals are responsible for egg production, vitellogenesis, ovulation, maintenance of the egg in utero, and oviposition/parturition (Callard et al., 1992; Custodia-Lora and Callard, 2002; Jones, 2017; Licht, 1979). Furthermore, hormones are required to induce tissue-level changes that facilitate pregnancy, including, for example, cellular changes in the uterus for nutrient transport to offspring. Finally, hormones facilitate communication between maternal and embryonic tissue throughout gestation, which is important for coordination of developmental features such as apposition of vascular beds within the placenta (Murphy et al., 2011).

The fundamental difference between oviparous and viviparous species is that in viviparous species eggs are retained in utero until development is complete. As embryonic development in ectotherms is temperature rather than time-dependent, viviparous mothers cannot precisely determine the developmental state of offspring without relying on embryonic cues. Hormone production by embryos therefore represents one possible way for eggs to be retained in utero and deposited at the correct developmental stage which would ultimately give rise to viviparity. For example, progesterone is a key hormonal regulator of pregnancy/gravidity in both mammals and reptiles (Custodia-Lora and Callard, 2002). Genes involved in progesterone biosynthesis have been identified in the chorioallantoic tissue of the chicken, suggesting that this tissue may ancestrally produce progesterone (Albergotti et al., 2009). Production of progesterone by the chorioallantoic placenta could play a significant role in the evolution of viviparity, because egg retention could be achieved in part by simply increasing the transfer of progesterone from embryo to mother by, for example, reducing eggshell thickness.

As well as facilitating maternal-offspring communication, embryonic production of hormones allows embryos to manipulate maternal physiology, which can facilitate parent-offspring conflict. Parent-offspring conflict is a major driver of the evolution of viviparity and placental functions in vertebrates (Blackburn, 2015; Crespi and Semeniuk, 2004; Zeh and Zeh, 2008). Conflict arises when offspring demand more resources than is optimal for the lifetime reproductive success of the parent (Trivers, 1974). For maternal-offspring conflict to occur during pregnancy, embryos must be able to manipulate maternal reproductive physiology, such as nutrient transport across the placenta. This manipulation can be achieved either by gaining direct access to maternal resources through invasive placentation, or through embryonic secretions that change the physiology of maternal tissue. In species with non-invasive epitheliochorial placentation, such as those examined in this study, nutrients must be actively transported across maternal tissue to the embryo, restricting embryonic control. However, embryonic control of transport could still occur in these species (Fowden et al., 2006): In the horse, which has non-invasive epitheliochorial placentation, embryos actively manipulate placental nutrient supply, likely by the production of hormones such as insulin like growth factor 2 (Allen et al., 2002).

In species with non-invasive placentation, if offspring are able to manipulate the activity of maternal tissues by the production and transport of hormones to maternal tissues, conflict may occur. Extensive embryonic production of growth factors occurs in the embryonic trophoblast of mammals (Fowden and Forhead, 2009; Fowden et al., 2006). Understanding how the production of growth factors correlates with different reproductive modes and the evolution of placentotrophy will allow us to understand if embryonic manipulation, through growth factor production, is a novel function of the chorioallantois in placentotrophic lineages. Alternatively, the production of growth factors may be the result of modifications to processes that occurred ancestrally in chorioallantoic tissue, with growth factors expressed in the chorioallantois of oviparous species.

We measured gene expression in the embryonic chorioallantoic tissue of species across the amniote phylogeny. To characterize the hormonal activity of the chorioallantoic membrane in amniotes we quantified the expression of genes which encode peptide hormones, proteins responsible for hormone synthesis, and hormone receptors. From these data we addressed two specific questions: 1) Is hormone production by the chorioallantoic placenta widespread in viviparous amniotes and 2) Has placental hormonal signaling evolved following the recruitment of hormone related genes in viviparous lineages, or is expression of hormone-related genes a result of coopting ancestral gene expression patterns.

2. Materials and methods

2.1. Species selection

Recent advances in next-generation sequencing technology make it possible to simultaneously measure the expression of most genes in a given tissue (transcriptome). We evaluated the expression of hormone-related genes in the CAM of multiple species using newly-generated and published transcriptome data for the chorioallantoic membrane (Fig. 2). We sampled two oviparous taxa including the chicken (*Gallus gallus*) and an oviparous population of

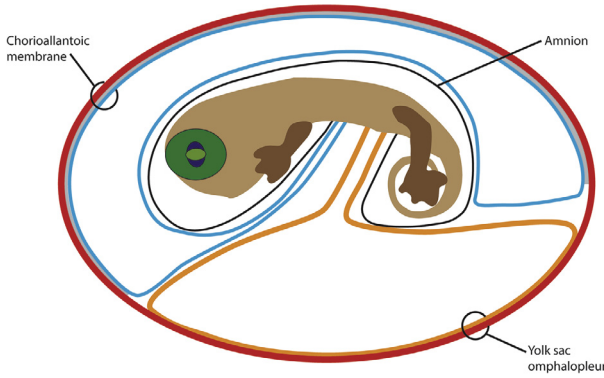

Fig. 1. Simplified diagram showing the generic layout of embryonic membranes in amniotes, modified from Ferner and Mess (2011). In oviparous taxa, these structures sit inside the eggshell membrane; in viviparous species the egg is maintained in the uterus. The shape, size, and layout of each membrane differs between amniotes (see Ferner and Mess (2011) for comparisons between major amniote groups). Red – embryonic ectoderm; grey –embryonic endoderm; blue – allantoic membrane; orange – yolk sac membrane.

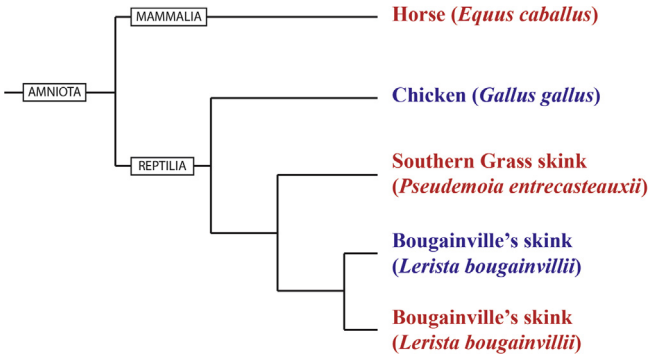

Fig. 2. Phylogenetic relationship of amniotes species used in this study. Viviparous taxa are denoted in red text, whilst oviparous taxa are denoted in blue.

the skink *Lerista bougainvillii* (which is a bimodally reproductive species). We sampled two separate origins of lizard viviparity in the skink *Pseudemoia entrecasteauxii* and a viviparous population of *L. bougainvillii.* The viviparous taxa differ in their mode of embryonic nutrition. *Lerista bougainvillii* is lecithotrophic—embryos rely on yolk for the vast majority of nutrients, and the simple placenta instead facilitates flux of water, ions, and gases. In contrast, *P. entrecasteauxii* is placentotrophic—embryos receive nutrients supplied by the placenta (and reduced yolk) via a relatively complex placenta with specializations for nutrient transport (Adams et al., 2005; Griffith et al., 2013a). Like most viviparous squamates, *P. entrecasteauxii* has non-invasive epitheliochorial placentation, meaning that the maternal and embryonic epithelia directly contact, but there is no breaching of the maternal tissue (Adams et al., 2005; Griffith et al., 2013b). To these data we added published mammalian transcriptome data for the CAM of the horse (Wang et al., 2012), representing a third origin of viviparity and a second origin placentotrophy in amniotes in our sample. The horse also possesses a non-invasive epitheliochorial placenta, making it possible to evaluate gene expression in two independent derivations of the same placental type in mammals and reptiles.

2.2. Measuring gene expression in chorioallantoic tissue

2.2.1. Chicken, Gallus gallus

We dissected chorioallantoic tissue from day 8 (n = 2) and day 18 (n = 3) chicken eggs in May 2012. To extract RNA, tissue was macerated using a mechanical homogenizer in 600 μL of Buffer RLT (QIAGEN) then homogenized using a QIAshredder spin column (QIAGEN). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). Extracted RNA was treated with Amplification Grade DNase 1 (Sigma-Aldrich). RNA quality was measured on the Agilent 2000 Bioanalyzer (Agilent Technologies) and was only used for transcriptome analysis if the RIN was greater than 8. Sequencing libraries were generated in house using the TruSeq RNA Sample Preparation kit (Illumina, Inc.), were pooled into lanes containing ten samples, and were sequenced on the HiSeq2000 (Illumina, Inc.). Raw transcriptome reads are available in the Sequenced Read Archive (Accession on acceptance). Gene counts for each gene were calculated by aligning raw sequencing reads to the complete cDNA library for *Gallus gallus* from Ensembl Release 78 (Cunningham et al., 2015) with Bowtie2 (version 2.0.0-beta7) (Langmead and Salzberg, 2012). We counted the number of transcripts that aligned to each cDNA contig with Samtools view (Li et al., 2009).

2.2.2. Southern grass skink, Pseudemoia entrecasteauxii

We collected chorioallantoic placental tissues from mid (embryonic stage 36/37 of the staging scheme used for *Zootoca vivipara*, n = 2) and late pregnant females (embryonic stage 40, which is the final developmental stage, n = 4, Dufaure and Hubert, 1961). RNA purification and sequencing was performed using the same methodology as the chicken. We collected a mean of $2.4 \times 10^7 \pm 3.8 \times 10^6$ sequenced reads per sample. Data analysis was performed using the same approach as the chicken except raw sequencing reads were aligned to a transcriptome for *P. entrecasteauxii* that was assembled *de-novo*.

We assembled a reference transcriptome for *P. entrecasteauxii de-novo* from the uterine tissue of early (n = 1) and late (n = 2) pregnant females, the embryonic chorioallantoic membrane (n = 2) and yolk sac membranes (n = 2) of late developing embryos, and adult brain tissue (n = 1). Transcriptomes were assembled with ABySS 1.3.4 (Simpson et al., 2009) from tissues of a single individual at a time, with kmer of 49, 59, and 69. After assembly, the transcriptomes from each sample were pooled creating a single assembled transcriptome from multiple tissues. Contigs in the transcriptome smaller than 100 bp and redundant contigs were removed with CD-HIT-EST (Huang et al., 2010) using default options. Contigs were identified by aligning against the *Anolis* proteome (Ensembl Build 70) using BlastX with an e-value of 10-5. The alignment rate of the raw reads to the assembled transcriptome was >90% for all samples. 60, 773 assembled transcripts were identified following blast to the *Anolis* proteome, which equates to 74% sequence coverage of protein coding genes in the published *Anolis* proteome. Unidentified contigs were further screened by aligning to a composite of the proteomes of human, chicken, opossum, platypus, zebra finch and Chinese tortoise (Ensembl Build 70) using the same criteria as above. A further 27, 431 transcripts were identified after blasting against additional proteomes.

2.2.3. Bougainville's skink, Lerista bougainvillii

We collected a gravid oviparous Lerista bougainvillii from the Burra Region of South Australia in 2011. This lizard was maintained in captivity until it laid eggs, and the CAM was dissected from one egg. Pregnant viviparous *L. bougainvillii* were collected on Kangaroo Island, South Australia in December of 2011 and 2012. Lizards were housed and then euthanized during late pregnancy, chorioallantoic placental tissue was dissected. All embryos were between developmental stage 35 and 40. RNA extraction and transcriptome sequencing was performed the same as for the chicken. We collected a mean of $2.9 \times 10^7 \pm 2.2 \times 10^6$ one hundered and one bp sequenced reads per sample. We used ABySS 1.3.4 to assemble all individual raw Illumina reads into larger contigs. Contigs in the transcriptome smaller than 100 bp and redundant contigs were removed with CD-HIT-EST (Huang et al., 2010) using default options. We identified the contigs using BLASTX with ten vertebrate reference genomes (Ensembl build 72). Finally, we used the short-read aligner Bowtie2 (version 2.0.0-beta7) (Langmead and Salzberg, 2012) to align the original raw Illumina reads to the identified contigs.

2.2.4. Horse, Equus callabus

Raw 40 bp single end Illumina reads from horse chorioallantoic girdle were taken from Wang et al. (2012). Four samples sequenced on the Illumina Genome Analyzer IIx were included, and had a mean of $3.1 \times 10^7 \pm 6.2 \times 10^5$ sequenced reads per transcriptome. We aligned raw sequenced reads to the complete *Equus callabus* cDNA collection in Ensembl Release 78 (Cunningham et al., 2015) with Bowtie2 (version 2.0.0-beta7) (Langmead and Salzberg, 2012). We counted the number of transcripts that aligned uniquely to each cDNA contig with Samtools view (version 0.1.18) (Li et al., 2009).

2.3. Identifying expressed genes

Although transcription occurs in specific parts of the genome, deep sequencing by RNA-seq will also identify small numbers of

transcripts from genes that are actively repressed (see Wagner et al. (2013) for more details). We used the transcript per million (TPM) metric to separate "expressed genes" from genes which had some sequenced transcripts but are unlikely to be genuinely regulated (Wagner et al., 2012, 2013). In cases where multiple assembled cDNA sequences existed for a single gene, gene counts from these transcripts were summed and this summed transcript number was divided by the summed contig length. Genes that had a mean TPM value greater than four were considered expressed; this cut off is consistent with the chromatin state of each gene (Hebenstreit et al., 2011). Our cut off is conservative and can confidently identify genes that are transcriptionally active, and not actively repressed (Wagner et al., 2013). We report all gene expression values in TPM.

2.4. Hormone-related genes

We compiled a gene list from the DAVID bioinformatics resource, the GeneCards Human Gene Database, and additional genes from readings in the literature to identify hormone-related genes for this analysis. Gene ontology terms (GO terms) for biological processes were downloaded from the DAVID bioinformatics resources 6.7 (Huang et al., 2009). All GO terms for biological processes containing a set of keywords (Supplementary Table 1) were collated. Gene symbols associated with these GO terms were extracted using a custom perl script using the data associated with DAVID IDs. Gene symbols were extracted from the GeneCards Human Gene Database (Version: 3.12.316 2 Feb 2015), by searching for genes that contained the word 'hormone' or the word 'growth factor' in their symbol, alias or identifier. In addition, a list of all Cytochrome P450 genes collected from GeneCards Human Gene Database (Version: 3.12.316 2 Feb 2015) were included, as this group of genes is involved in steroid metabolism (Lewis, 1996). Other genes were added if they were reported in the literature to be of importance to the regulation of hormone production or metabolism.

3. Results and discussion

3.1. CAM hormone gene expression

The CAM is a hormone-producing organ in amniotes: of the 423 hormone-related genes examined, 321 genes are expressed in the CAM of at least one of the studied species. Expression of hormone-related genes is not confined to viviparous taxa, which confirms findings previously reported in oviparous birds, crocodiles, and tortoises (Albergotti et al., 2009; Cruze et al., 2012, 2013). Ninety-three genes show conserved expression across all studied species, suggesting they may perform essential functions of the chorioallantoic tissues in amniotes. However, it is possible that the physiological function of these proteins has been modified in viviparous lineages to perform novel pregnancy-associated roles. No genes were expressed only in viviparous species, suggesting that the evolution of viviparity does not require *de novo* recruitment of any particular hormone-related genes to placental tissues. From our survey, only one gene exhibited expression specific to placentotrophic species, although ten genes have lost their expression in placentotrophic lineages, suggesting a potential role of gene expression loss in the evolution of placentotrophy. In the remainder of the discussion, we concentrate on expression of genes related to specific groups of hormones that are important in amniote reproduction.

3.2. Steroid hormone synthesis

We examined the expression of 47 genes involved in steroid hormone synthesis and metabolism (Table 1). Of these genes, six are expressed in all species, with four of these belonging to the cytochrome P450 family of proteins (CYP). CYPs are a diverse group of enzymes that are involved in cholesterol and steroid synthesis and metabolism (Werck-Reichhart and Feyereisen, 2000). Although the specific functions of the encoded proteins of these genes are largely unknown, they may play essential roles in the CAM. The newly-sequenced reptile species differ from the Alligator, which expresses specific genes in the CAM involved in androgen and estrogen synthesis (*CYP17A1* and *CYP19A1* respectively) (Cruze et al., 2012).

3.2.1. Progesterone

The defining feature of a viviparous species is that embryos are maintained in the mother until development is complete (Blackburn, 2006). Progesterone is a regulator of gravidity/pregnancy in all studied amniotes and so was a hormone of interest in our study. In turtles, increased progesterone production is associated with ovulation (Guillette et al., 1991; Licht et al., 1982), and prevents oviposition (Klicka and Mahmoud, 1977; Mahmoud et al., 1988). In the relatively short reproductive cycle of chickens (26 h), progesterone peaks just prior to ovulation (Laguë et al., 1975). In oviparous squamates, progesterone is produced by the corpus luteum and hormone concentration is correlated with gravidity (the holding of eggs) and normally declines prior to oviposition (Díaz et al., 1994; Fox and Guillette, 1987). Typically in viviparous amniotes, progesterone is maintained at relatively high concentrations and decreases prior to parturition (Bazer, 1992; Fergusson and Bradshaw, 1991; Murphy and Thompson, 2011). During eutherian mammal pregnancy, progesterone is produced by ovarian *corpora lutea* until the embryonic placenta has developed sufficiently to take over hormone production (Stocco et al., 2007; Tuckey, 2005). The pattern of shifting progesterone production from the *corpus luteum* to the chorioallantois during pregnancy also occurs in the viviparous lizard *Chalcides chalcides* (Guarino et al., 1998).

For embryonic progesterone production to alter the length of pregnancy, maternal tissues must be receptive to the progesterone signal. Uterine receptivity to progesterone occurs via the progesterone receptor (*PGR*), which is expressed in the uterus of the live bearing lizards *Chalcides ocellatus* and *P. entrecasteauxii* (Biazik et al., 2012; Brandley et al., 2012; Griffith, 2015). Uterine PGR production has therefore occurred in amniote reproduction since before the split of these two amniote lineages ~300 million years ago.

Progesterone production occurs by first transporting cholesterol to the mitochondrion (typically by steroidogenic acute regulatory protein; StAR), where it is transported to the inner mitochondrial membrane by StAR-related lipid transfer domain protein 3 (StARD3), and converted to pregnenolone by cytochrome P450, family 11, subfamily A, polypeptide 1(CYP11A1). Finally, pregnenolone is converted to progesterone by 3 beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase type I (HSD3B1) (Fig. 3; Payne and Hales, 2004).

Expression of genes encoding each enzyme in the progesterone synthesis pathway occurs in the horse placenta (Table 1), but not all are expressed in the chorioallantoic tissues of the other studied species. *STARD3* is expressed in all the taxa we examined; as STARD3 is important for mobilization of cholesterol in the mitochondria, this step in the progesterone synthesis pathway is common in the synthesis and metabolisation of many steroid hormones (Payne and Hales, 2004). In humans (and potentially chickens) production of STARD3 is sufficient to facilitate cholesterol mobilization for progesterone synthesis (Tuckey, 2005). Chickens lack expression of *STAR*, which facilitates mobilization of cholesterol to the mitochondria, similarly STAR is not produced in the human trophoblast despite production of progesterone in this tissue (Strauss et al., 1996). CYP11A1 and HSD3B1 are necessary for the production of proges-

Table 1
Expression patterns of genes involved in steroid synthesis and metabolisation in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts per gene, corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n = 5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n = 6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n = 1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n = 4)	Horse, *Equus caballus* (n = 4)	Pattern
Genes involved in progesterone synthesis						
STARD3	**15.5 ± 1.4**	**22 ± 2**	**22.9**	**21.1 ± 1.1**	**10.9 ± 0.6**	All
CYP11A1	**20.3 ± 2.6**	–	0	0.2 ± 0.2	**115.9 ± 79.7**	
HSD3B1	**44.6 ± 15.9**	–	0.2	0.2 ± 0.1	**74 ± 9.9**	
STAR	0.9 ± 0.1	0.4 ± 0.2	**44.8**	3.4 ± 1.3	**72.9 ± 34**	
Steroid hormone receptors						
AR	**22.7 ± 6.3**	0.9 ± 0.3	**107.5**	**140.3 ± 15.2**	0.3 ± 0.2	Lecithotrophic
PGR	1.1 ± 0.6	3.1 ± 0.3	**12.4**	**14.2 ± 1.5**	0.2 ± 0.1	
ESR1	0.2 ± 0.1	0.2 ± 0.1	1.6	2.2 ± 0.1	–	
ESR2	0.1 ± 0	–	0	0.6 ± 0.3	–	
CYP20A1	**24.6 ± 2.1**	**89.3 ± 7.5**	**19**	**19.7 ± 2.1**	**14.4 ± 3.3**	All
CYP27A1	**9.1 ± 1.4**	**711.4 ± 79.4**	**19.9**	**13.1 ± 1.9**	**5.1 ± 2.9**	All
CYP39A1	**15.1 ± 2.7**	**5.1 ± 2.1**	**24.7**	**22.5 ± 2.3**	**13.6 ± 2.4**	All
CYP51A1	**25.5 ± 5.5**	**385.4 ± 103.3**	**29**	**84.4 ± 6.3**	**102.1 ± 16.3**	All
NR3C1	**21.2 ± 2.2**	**39.4 ± 6**	**75.5**	**77.7 ± 10.4**	**7.5 ± 1.2**	All
CYP26B1	**4.9 ± 0.5**	0.3 ± 0.3	**18.3**	**33.7 ± 11.5**	0.2 ± 0.1	Lecithotrophic
CYP46A1	**18.5 ± 3.8**	–	**39.6**	**28.2 ± 8.3**	0.1 ± 0.1	Lecithotrophic
CYP4F22	**30.7 ± 4.3**	–	**164.4**	**62.5 ± 9**	0.1 ± 0.1	Lecithotrophic
CYP2U1	2.9 ± 0.3	**5.3 ± 0.8**	**14.6**	**21.9 ± 5.5**	1.2 ± 0.5	Lizard
CYP7B1	3.4 ± 0.4	**30.3 ± 12.5**	**18.4**	**25.8 ± 5.8**	0.2 ± 0.1	Lizard
CYP2D6	**22.5 ± 2.2**	**28.8 ± 3.5**	**65.9**	**120.6 ± 22.1**	–	Reptile
CYP2R1	**5.2 ± 0.5**	**7.5 ± 1.4**	**7**	**7.6 ± 1.3**	0.2 ± 0.1	Reptile
CYP4V2	**7.4 ± 1.9**	**5.5 ± 1.2**	**68.2**	**63.6 ± 15.9**	0 ± 0	Reptile
CYP17A1	0.4 ± 0.1	1.7 ± 0.5	1	0.3 ± 0.2	**1918.6 ± 1310.5**	
CYP19A1	0.7 ± 0.1	1 ± 0.7	–	–	**1572 ± 574.7**	
CYP1A1	0.5 ± 0.1	–	**87.2**	**114.3 ± 15.2**	**4.3 ± 4.3**	
CYP1A2	–	–	–	–	0.3 ± 0.3	
CYP1B1	3.9 ± 0.9	**5.4 ± 1**	**94.5**	**38.7 ± 2.1**	**29.6 ± 14.7**	
CYP21A2	2.2 ± 0.8	–	–	–	0.4 ± 0.3	
CYP24A1	0.8 ± 0.4	–	**4.4**	**55.7 ± 21.4**	3.2 ± 1.3	
CYP26A1	1.1 ± 0.5	0.3 ± 0.2	–	–	**6.8 ± 2.4**	
CYP26C1	0.3 ± 0.1	**4.5 ± 0.8**	**5.8**	3.5 ± 1.2	1.4 ± 0.6	
CYP27B1	–	1.7 ± 0.4	–	–	0 ± 0	
CYP27C1	1.5 ± 0.5	–	–	–	0.2 ± 0.1	
CYP2A13	–	–	**1129.7**	**517.5 ± 151.8**	–	
CYP2A6	–	–	**396.9**	**31.3 ± 17.6**	–	
CYP2A7	–	–	**317.6**	**16.1 ± 7**	–	
CYP2B6	–	–	**4**	**14.6 ± 5.1**	–	
CYP2F1	–	1.2 ± 0.2	**11.8**	**9.3 ± 4.4**	0.1 ± 0.1	
CYP2J2	–	**18.9 ± 1.7**	**49.1**	**166.2 ± 41.9**	**4.7 ± 2.2**	
CYP2S1	–	–	–	–	**6.8 ± 0.7**	
CYP2W1	–	–	**7.2**	**47.7 ± 29.3**	0.7 ± 0.4	
CYP3A7	–	–	**208**	**11.5 ± 4.9**	–	
CYP4F3	–	–	**766.9**	**279.8 ± 47.7**	–	
CYP4F8	–	–	–	–	1.1 ± 0.7	
CYP4X1	–	–	–	–	0 ± 0	
CYP7A1	2.8 ± 0.2	–	–	–	0.5 ± 0.2	
CYP8B1	–	–	**16.7**	**18.4 ± 2.4**	0 ± 0	
NR5A1	0.2 ± 0.1	0.1 ± 0.1	–	–	–	

terone from cholesterol (Payne and Hales, 2004). The presence of both enzymes in chickens is consistent with progesterone production during development (Albergotti et al., 2009). In *P. entrecasteauxii HSD3B1*and *CYP11A1* are absent from the reference transcriptome of this species, so it is not possible to say with confidence that these genes are not expressed. In *L. bougainvillii*, both *CYP11A1* and *HSD3B1* are present in the reference transcriptome (so our approach could reliably detect the expression of these genes) but these genes are not expressed in the chorioallantoic membrane of either oviparous or viviparous populations. This result strongly suggests that in some viviparous lizards the embryonic component of the placenta is not producing progesterone via any known synthesis pathway. As expression of these genes in the CAM is absent in *L. bougainvillii*, embryonic progesterone production by this pathway is not simply an ancestral trait of amniotes that has been utilized during the evolution of all instances of viviparity. Our results do not preclude the possibility that the chorioallantoic membrane of skinks synthesizes progesterone by an unknown pathway. However, our findings suggest that placental production of progesterone in viviparous amniotes is not the result of a conserved ancestral gene expression pattern.

3.2.2. Steroid hormone receptors

Androgen receptor (AR) binds steroid hormones, including testosterone, and participates in the development of male characteristics, but it is also important to female physiology (Cloke and Christian, 2012). AR is expressed in female reproductive tissues, and in humans its expression fluctuates through cycling of the endometrium (Barad et al., 2007). Androgens have been identified in mammalian trophoblast, and increased AR levels are associated with pre-eclampsia (Hsu et al., 2009). Androgen receptor is highly expressed in all lecithotrophic taxa we examined, but is not

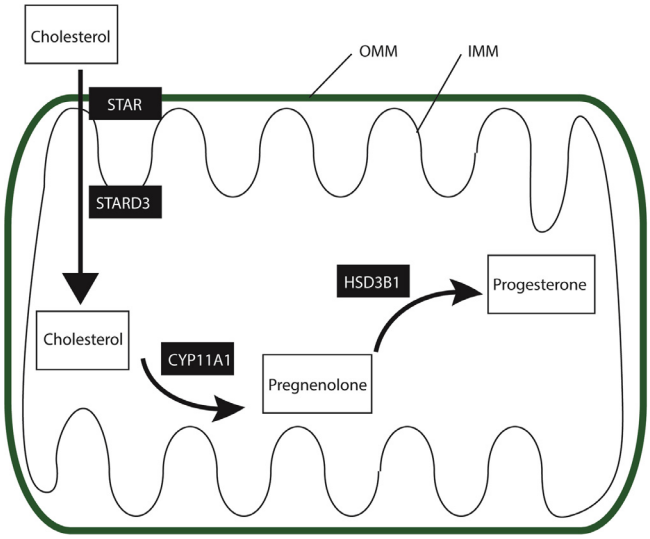

Fig. 3. Progesterone synthesis pathway, which occurs inside the mitochondrion pictured (green). Arrows indicate movement or modification of steroid hormones (empty boxes). Black boxes indicate enzymes responsible for mobilization or movement of hormones at each step. OMM – outer mitochondrial membrane; IMM – inner mitochondrial membrane; STAR – steroidogenic acute regulatory protein; STARD3 – StAR-related lipid transfer domain containing 3; CYP11A1 – cytochrome P450, family 11, subfamily A, polypeptide 1; HSD3B1 – 3 beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase type I.

expressed in placentotrophic taxa; horse *and P. entrecasteauxii* (Table 1). The presence of maternally produced hormones (such as androgens) could negatively impact offspring development, in particular by affecting sexual characteristics (Uller and Olsson, 2003). In experiments where testosterone concentrations have been artificially increased during development, offspring have reduced embryonic growth rate, compromised immune function, and altered behavior (Sinervo et al., 2000; Uller and Olsson, 2003). Our results suggest that by decreasing the expression of androgen receptor in species with high levels of placental transport, embryos may decrease the effect of maternally-derived androgens on embryonic development.

The progesterone receptor gene (*PGR*) is only expressed in the CAM of both oviparous and viviparous populations of *L. bougainvillii* (Table 1), suggesting that receptivity to progesterone in the CAM may be absent in most non-mammalian amniotes and is not necessary for viviparity. As the CAM of *L. bougainvillii* expresses *PGR*, but does not express the genes necessary for progesterone synthesis, we suggest that progesterone may play a different role during reproduction in these lizards. Further work is necessary to identify if progesterone is necessary for the maintenance of pregnancy in *P. entrecasteauxii* and *L. bougainvillii*, and to confirm where it is produced.

Estrogen is an important hormone for the regulation of the female reproductive system in reptiles and mammals (Callard et al., 1972; Katzenellenbogen and Greger, 1974; Wang et al., 2000). Estrogen receptors are not expressed in the CAM of any studied species (Table 1), suggesting that estrogen does not regulate growth of the chorioallantoic membrane in oviparous or viviparous species.

3.3. Growth factors and peptide hormones

3.3.1. IGF2

Insulin like growth factor 2 (IGF2) is a key regulator of placental growth and function in mammals, and plays an important role in parent offspring conflict. Whilst embryos (which inherit half of their genome from their fathers) seek to maximize their own fitness, mothers seek to maximize their lifetime reproductive success, resulting in conflict over how resources should be provisioned between individual offspring and future reproductive events (Trivers, 1974). The maternal allele of *IGF2* is silenced by genomic imprinting in livebearing mammals. Imprinting of this gene occurs because of parent-offspring conflict, whereby placental production of IGF2 facilitates embryonic manipulation of maternal nutrient provisioning (Haig, 2000; Wilkins and Haig, 2003). Under-expression of IGF2 results in decreased growth of mouse embryonic and placental tissues, whilst increased IGF2 abundance in utero results in offspring overgrowth in humans, mice and sheep (Coan et al., 2008; Fowden and Forhead, 2009). IGF2 works as a signal of fetal demand, regulates fetal growth, and interacts with the expression of nutrient transport proteins in placental tissues (Constância et al., 2005; Reik et al., 2003; Sibley et al., 2004). Whilst genomic imprinting of this gene appears to be absent in at least some viviparous reptiles, placental production of this gene could antagonize parent offspring conflict (Griffith et al., 2016). The production of IGF2 by offspring during gestation also occurs in live-bearing teleost fishes, where ongoing parent-offspring conflict has driven selection on the gene, likely because of its ability to regulate placentotrophy (O'Neill et al., 2007).

IGF2 is expressed in the CAM of all studied species, regardless of reproductive mode (Table 2). Ancestral expression of *IGF2* in the CAM suggests that this protein may be a regulator of growth and proliferation of the embryo in oviparous taxa. Uterine expression of the receptor for IGF2, the insulin growth factor like receptor 1, is ancestral to mammals and present in the uterine tissue of *P. entrecasteauxii* (Griffith, 2015; Lynch et al., 2015). During the evolution of viviparity, reduced eggshell thickness and the apposition of maternal and fetal tissues would allow embryonic IGF2 to diffuse or be transported to maternal tissues, which suggests that placental production of IGF2 is actually an exaptation that has been utilized to manipulate maternal tissues during the evolution of viviparity. Ancestral expression of IGF2 in the CAM, may explain why the consequences of parent offspring conflict can manifest in species that have only recently evolved viviparity (Blackburn, 2015; Van Dyke et al., 2014b).

3.3.2. Angiogenic factors

In conjunction with its ability to regulate placental nutrient transfer, IGF2 can regulate endothelial cell migration (Herr et al., 2003). Changes in uterine angiogenesis are essential for the evolution of viviparity to support increasing embryonic oxygen demand during pregnancy (Parker et al., 2010a). Increased vascularization during pregnancy results in gross morphological changes that are necessary for placental functions (Parker et al., 2010a,b). Embryonic regulation of vascularization occurs in some viviparous squamates, where the vascular bed of the mother is aligned with the position of the embryo inside the egg, even in cases where the embryo is oriented obliquely to the uterus (Murphy et al., 2010). A number of genes encoding proteins with angiogenic potential show consistent expression in the CAM across all studied species (Table 3). Of particular note are VEGFA and PGF, which are the most well studied genes of uterine angiogenesis (Autiero et al., 2003; Danastas et al., 2015; Murphy et al., 2010; Torry et al., 2003; Whittington et al., 2015a). No genes show a viviparity-specific expression pattern, suggesting that uterine angiogenesis during pregnancy has not evolved by recruiting gene expression to the embryonic membranes. Embryonic signals to increase uterine vasculature may occur by simply utilizing proteins already expressed in these tissues.

3.4. Thyroid hormones

Thyroid hormones are essential for normal embryonic development, especially of nervous tissue, and are regulators of metabo-

Table 2
Expression patterns of growth factors and growth factor receptors in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts for per gene, corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n = 5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n = 6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n = 1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n = 4)	Horse, *Equus caballus* (n = 4)	Pattern
IGF1R	**14.8 ± 1.8**	**29.8 ± 4.2**	**25.7**	**19.1 ± 3.4**	**38.8 ± 16.1**	All
IGF2	**59.6 ± 7.3**	**15.1 ± 2.3**	**17.9**	**34.3 ± 8.2**	**1901.3 ± 302.5**	All
PDGFB	**60.5 ± 14.6**	**20 ± 2.7**	**8.5**	**17.2 ± 3.7**	**6.4 ± 5.2**	All
PDGFC	**9.6 ± 0.6**	**39.2 ± 6.9**	**10.5**	**18.8 ± 3.6**	**31.9 ± 6.6**	All
PPAP2B	**117.7 ± 18.4**	**37.3 ± 3.3**	**33.4**	**56 ± 4.5**	**16.7 ± 1.9**	All
PSIP1	**29.7 ± 3**	**37 ± 2.7**	**19.6**	**29.1 ± 4.4**	**43 ± 9**	All
FGF20	**4.8 ± 0.6**	–	–	–	3.9 ± 1.3	Chicken
FGF22	**11.9 ± 4.3**	–	–	–	–	Chicken
FGF23	**17.8 ± 1.7**	–	–	–	0 ± 0	Chicken
INHBA	**17.9 ± 2.4**	0.5 ± 0.2	**4.2**	**10 ± 4.7**	0.7 ± 0.2	Lecithotrophic
KITLG	**6.1 ± 0.7**	–	**23.9**	**14.5 ± 1.6**	0.9 ± 0.4	Lecithotrophic
HGF	–	**5.9 ± 0.8**	**17.9**	**19.9 ± 1.7**	0.4 ± 0	Lizard
NGF	2.4 ± 0.5	**14.5 ± 3.3**	**10**	**6.2 ± 0.9**	0 ± 0	Lizard
AREG	**71.1 ± 17.6**	–	**5.1**	3.2 ± 0.7	1.4 ± 0.9	Oviparous
AMH	1 ± 0.3	–	–	–	–	
BMP15	2.6 ± 0.5	3.6 ± 0.8	–	–	–	
BMP6	**153.2 ± 29.1**	**21.5 ± 1.8**	**24.9**	**38.9 ± 3.5**	1.3 ± 0.4	
CLEC11A	–	1 ± 0.2	–	–	–	
FGF11	–	–	–	–	0.2 ± 0.1	
FGF12	**29.5 ± 3.8**	2.5 ± 0.3	2.5	**45.5 ± 38.8**	0 ± 0	
FGF13	0.6 ± 0.1	–	**40.4**	**127.9 ± 22.5**	0.4 ± 0.1	
FGF14	0.6 ± 0.2	0.7 ± 0.2	–	–	–	
FGF16	1.3 ± 0.7	1 ± 0.6	2.3	0.7 ± 0.2	0.1 ± 0.1	
FGF19	0.7 ± 0.2	1 ± 0.7	**8.7**	**34.2 ± 10**	0.2 ± 0.2	
FGF21	–	0.6 ± 0.6	**33.5**	1.2 ± 0.6	0 ± 0	
FGF3	0.3 ± 0.1	–	–	–	0.2 ± 0.2	
FGF4	0 ± 0	–	–	–	0 ± 0	
FGF5	2.5 ± 0.2	–	–	–	–	
FGF7	2.3 ± 0.3	3.7 ± 1.1	2.8	**18.4 ± 6**	**55.3 ± 17.7**	
GDF10	3.9 ± 1.5	–	**7.3**	**33.7 ± 6.8**	0 ± 0	
GDF11	–	0.2 ± 0.2	–	1.7 ± 0.3	**13.7 ± 4.2**	
GDF15	–	–	**26.6**	**62.7 ± 17.3**	–	
GDF2	–	–	–	–	0.1 ± 0.1	
GDF5	3.3 ± 0.9	**17.9 ± 2.3**	–	–	0.1 ± 0.1	
GDF6	–	–	**21**	**26.8 ± 4.2**	**4.2 ± 1.1**	
GDF7	–	**12.4 ± 2.5**	1.4	**5 ± 1.2**	–	
GDF9	–	–	–	–	0.1 ± 0.1	
HBEGF	**43.4 ± 15.9**	**46.1 ± 1.8**	3.8	**6.6 ± 0.8**	2.8 ± 0.8	
HDGF	–	–	**1335.8**	**1187.1 ± 289.9**	**179.6 ± 22.1**	
HDGFRP3	–	–	**38.1**	**65.4 ± 5.6**	–	
IGF1	–	0.2 ± 0.1	–	–	0.2 ± 0.1	
IGF2R	**13.3 ± 2.6**	–	**318.7**	**209.7 ± 27.3**	**70.2 ± 6.2**	
IL4	0 ± 0	–	–	–	–	
IL6	3.7 ± 1.1	–	–	–	0 ± 0	
IL9	1.8 ± 0.9	–	–	–	–	
LEFTY2	0 ± 0	–	**74.2**	**256.4 ± 37.7**	–	
MDK	**900.5 ± 268.6**	**128.7 ± 18.5**	–	–	–	
MST1	**16.3 ± 1.6**	–	–	–	**109.3 ± 18.7**	
MSTN	–	–	–	–	0.1 ± 0.1	
NODAL	0.2 ± 0.1	0.6 ± 0.2	–	–	0.2 ± 0.1	
NOV	**28.7 ± 6.8**	0.9 ± 0.4	**2823.2**	**2664 ± 385.9**	**20.3 ± 12.1**	
NRG1	0.5 ± 0.1	0.5 ± 0.1	**11.8**	**9.2 ± 1.1**	0.8 ± 0.2	
NTF3	**30.1 ± 5.5**	**11.5 ± 1.5**	**29.3**	**17 ± 2.1**	1.1 ± 0.4	
PDGFD	**62 ± 10.6**	**30.4 ± 8.6**	33	**25.7 ± 9.3**	1.8 ± 0.6	
PTN	**39.4 ± 9.7**	0.5 ± 0.2	3.4	**6.3 ± 3.5**	**35 ± 11.5**	
TDGF1	–	–	–	–	0.1 ± 0.1	
TGFB1	–	**23.6 ± 9.2**	**14.7**	**14.2 ± 1.9**	**12.2 ± 4.4**	
TGFB3	**176.8 ± 25.7**	–	3.5	**7 ± 1.1**	0.5 ± 0.2	
VEGFB	–	–	–	–	**71.4 ± 14.8**	
VGF	–	0.2 ± 0.1	–	–	–	

lism and developmental rate (Burrow et al., 1994; Morreale de Escobar et al., 2004). Type 3 iodothyronine deiodinase (DIO3) metabolizes thyroid hormones in the placental tissues of mammals by deiodinating thyroxine (T_4) and tri-iodothyronine (T_3) to produce inactive metabolites (Chan et al., 2009; Roti et al., 1981). Of the 463 genes examined, only *DIO3* is present in the reference transcriptomes of all studied species, and is uniquely expressed in the CAM of placentotrophic species (Table 4). As thyroid hormone levels must be tightly regulated for proper embryonic development, expression of *DIO3* in the CAM may be an embryonically regulated mechanism to limit the exposure of the fetus to maternal thyroid hormone (Galton et al., 1999).

Type 3 iodothyronine deiodinase is mostly a paternally-expressed gene in therian mammals, although it is incompletely imprinted and imprinting differs between tissues and developmental stages (Martinez et al., 2014). Preferential paternal

Table 3
Expression patterns of genes involved in angiogenesis in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts for per gene, corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n = 5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n = 6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n = 1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n = 4)	Horse, *Equus caballus* (n = 4)	Pattern
CTGF	**113.2 ± 27**	**57.4 ± 8**	**59**	**90.3 ± 6.9**	**40.4 ± 16.1**	All
CYR61	**75.8 ± 5**	**64.6 ± 5.9**	**40.5**	**57.3 ± 6.5**	**56.1 ± 24.9**	All
EGFL7	**190.4 ± 26.9**	**74.1 ± 4.4**	**227.6**	**405 ± 51**	**4.7 ± 1.3**	All
MAP3K7	**69.6 ± 4.3**	**45.2 ± 3.1**	**117.4**	**103 ± 2.7**	**11.9 ± 1.9**	All
NRP1	**50.9 ± 4.3**	**22.6 ± 1.8**	**43**	**59.4 ± 12**	**6.4 ± 0.7**	All
NRP2	**55.4 ± 5.8**	**19 ± 0.8**	**18.2**	**24 ± 3**	**6.6 ± 1.1**	All
PGF	**108 ± 41.7**	**43 ± 1.6**	**35.1**	**68.8 ± 10.7**	**6.1 ± 5.5**	All
VEGFA	**33.5 ± 6.7**	**1433.8 ± 168.3**	**146.1**	**134.4 ± 11**	**49 ± 20.7**	All
FGF9	**19.4 ± 1.8**	3.2 ± 1	0.6	2.6 ± 0.5	–	Chicken
FGF2	–	**7.4 ± 0.9**	**4.9**	**6.1 ± 0.8**	0.1 ± 0.1	Lizard
PDGFA	–	**15.5 ± 2.3**	**45.2**	**11.2 ± 2.8**	–	Lizard
ANGPTL6	0 ± 0	**68.6 ± 6**	**9.5**	**45.4 ± 11.2**	**12.8 ± 10.8**	
CXCL12	**390.5 ± 69.1**	**19.9 ± 8.4**	**20.7**	**25.8 ± 5.5**	0.9 ± 0.4	
EGF	0.1 ± 0	0.2 ± 0.1	0	0.2 ± 0	0.1 ± 0	
FGF1	**4.5 ± 1.6**	1.7 ± 0.4	**4.5**	3.8 ± 1.2	**4.4 ± 0.8**	
FGF10	1.3 ± 0.3	–	–	–	1.8 ± 0.4	
FGF18	0.1 ± 0	0.3 ± 0.1	1	3.7 ± 1.1	0.1 ± 0.1	
FGF6	0 ± 0	–	–	–	–	
FIGF	**28 ± 9.3**	**39 ± 8.7**	**8.8**	**31.2 ± 4**	0.1 ± 0.1	
FLT1	**6.4 ± 0.6**	**67.4 ± 9**	**12.5**	**9.2 ± 1.8**	0.5 ± 0.1	
KDR	**32.2 ± 4.6**	**57.3 ± 8.1**	**13.3**	**22.2 ± 3**	1.5 ± 0.1	
PROK1	3.3 ± 0.5	**68.6 ± 8.6**	–	–	–	
SHH	1.3 ± 0.2	0.3 ± 0.1	–	–	0.4 ± 0.2	
TGFA	3.2 ± 0.4	–	**47.2**	**44.7 ± 9.8**	1 ± 0.2	
TGFB2	**26 ± 3**	–	**6**	**12 ± 1.7**	**4.7 ± 0.9**	
TGFBR2	**42.8 ± 4.5**	**92.1 ± 12.4**	**77.5**	**95.9 ± 6.2**	–	
TNFRSF12A	–	**35.9 ± 7.5**	**33.2**	**48.3 ± 8.1**	**77.6 ± 14.9**	
TYMP	–	–	**142.4**	**139.2 ± 20.5**	**35.4 ± 3.3**	
VEGFC	**18.9 ± 1.4**	**10.7 ± 1.1**	**28.5**	**39.4 ± 4.5**	1.2 ± 0.6	

expression of this gene suggests that it imposes different fitness implications depending on the parent from which it is inherited (Haig, 2000; Rocha et al., 2008). Typically this is the result of conflict over resources, whereby fathers (by means of the paternally inherited genome) manipulate mothers into providing more nutrients to the embryos than is optimal for the mother's life time

Table 4
Expression patterns of genes involved in thyroid hormone metabolism and receptor binding in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts for per gene, corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n = 5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n = 6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n = 1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n = 4)	Horse, *Equus caballus* (n = 4)	Pattern
Thyroid hormone metabolic process						
DIO3	0	**1310.5 ± 330**	0.60	2.3 ± 2.1	**16.8 ± 6.1**	Placentotrophic
MED1	**22.2 ± 1.5**	**10.5 ± 1.5**	**24.40**	**20.2 ± 2.1**	**6 ± 1.2**	All
DIO1	**24.3 ± 4.3**	–	–	–	–	Chicken
CGA	–	1.3 ± 1	–	–	**8432.2 ± 2800**	Horse
CRYM	**26.2 ± 2.9**	**50.9 ± 5.9**	**111.70**	**126.7 ± 13.4**	1.5 ± 0.4	Reptile
SULT1B1	**82.3 ± 11.6**	**4.7 ± 1.5**	**33.60**	**48.8 ± 2.6**	–	Reptile
DIO2	**22.7 ± 4.2**	**402.5 ± 73**	1.10	2.7 ± 0.7	0 ± 0	
Thyroid hormone receptor binding						
HMGN3	**562.8 ± 18**	**75 ± 4.1**	**323.40**	**322.2 ± 19.2**	**23.1 ± 4.6**	All
JMJD1C	**13.4 ± 2**	**16.9 ± 2.9**	**23.30**	**18.4 ± 3.8**	**7.4 ± 1.7**	All
MED1	**22.2 ± 1.5**	**10.5 ± 1.5**	**24.40**	**20.2 ± 2.1**	**6 ± 1.2**	All
MED12	**13.2 ± 1.1**	**24.9 ± 3.9**	**23.00**	**14.8 ± 2.2**	**28.2 ± 6.8**	All
MED13	**9 ± 0.3**	**16.7 ± 2.6**	**19.20**	**22.5 ± 3.7**	**7.5 ± 2.2**	All
MED14	**15.5 ± 0.8**	**41.7 ± 2.6**	**11.80**	**12.2 ± 1.4**	**7.9 ± 1.7**	All
MED16	**14.3 ± 1.1**	**26.3 ± 3.4**	**10.40**	**12.5 ± 1.4**	**17.1 ± 2.2**	All
MED17	**23.4 ± 1**	**16.8 ± 1.3**	**100.90**	**125.8 ± 15.2**	**7.1 ± 0.7**	All
MED24	**20.5 ± 1.6**	**14.1 ± 1.1**	**21.90**	**18.3 ± 0.7**	**7.7 ± 1.1**	All
NCOA3	**8.4 ± 2**	**36.6 ± 5**	**17.40**	**17.7 ± 2**	**17.4 ± 5.8**	All
NCOA6	**14.9 ± 2.1**	**32.1 ± 4**	**21.70**	**13.8 ± 2.2**	**11.5 ± 3.3**	All
THRAP3	**84.8 ± 4.3**	**63.5 ± 4.5**	**104.30**	**84.2 ± 6.5**	**79.8 ± 13.1**	All
TRIP12	**30.5 ± 2.8**	**78.4 ± 11.3**	**156.80**	**115 ± 16.1**	**8.2 ± 1.7**	All
ZNHIT3	**57.2 ± 2.6**	**13.7 ± 2.1**	**24.60**	**34 ± 5.8**	**37 ± 2.3**	All
TRIP6	–	–	–	–	**4.5 ± 1.2**	Horse
MED30	**39.4 ± 1.8**	**15.7 ± 1.6**	–	–	**30.1 ± 1.2**	

reproductive success (Crespi and Semeniuk, 2004), but it is not yet clear how *DIO3* imprinting might influence placental nutrient transport.

The observed expression of genes involved in thyroid hormone receptor binding in oviparous and viviparous species (Table 4) suggests that the chorioallantoic tissue is ancestrally responsive to thyroid hormone; however, the role of thyroid hormone in the placentae of mammals and reptiles is poorly understood. The expression of *DIO3* in the chorioallantoic tissue of placentotrophic species observed here but no other taxa suggests that thyroid hormone may be important for the evolution of placental nutrient transport, but further work is required to identify the functional significance of these findings. In particular, it is necessary to identify if thyroid hormone is transported across the placenta to offspring during development in non-mammalian viviparous amniotes. Secondly, it is important to identify the role of *DIO3* in pregnancy, which is poorly understood in both reptiles and mammals.

4. Conclusions and future research

The conserved expression of hormone producing genes observed here suggests that the chorioallantoic membrane is ancestrally an endocrine organ. Ancestral expression of several hormones in all studied amniotes, including the angiogenic factors VEGFA and PGF, suggests that some placental signaling function has evolved by utilizing genes expressed in the CAM of the ancestral amniote. An important evolutionary question follows from our study: if many hormones important for placental functions in amniotes are the result of ancestral gene expression patterns, then would a placenta derived from a non-homologous tissue (such as the yolk sac placenta of marsupials or the seahorse placenta formed from an abdominal skin pouch) produce a different set of hormones (Renfree, 2010; Whittington et al., 2015b)?

Differences in the expression of specific genes between taxa allow us to understand how the endocrine properties of the CAM have changed with respect to the evolution of viviparity and placentotrophy. Given that no genes are solely expressed in viviparous species; our results suggest that the evolution of viviparity has not required new recruitment of any specific hormone-related gene. Instead, viviparity is likely to have evolved using genes that were ancestrally expressed in the CAM. Only one gene, *DIO3*, shows a placentotrophy-specific pattern of gene expression. *DIO3* is an imprinted gene in mammals and has been recruited to the placental tissues of *P. entrecasteauxii* and the horse. As *DIO3* is preferentially paternally expressed, it may act to increase offspring resource uptake during pregnancy, although the mechanisms by which this might occur are unclear.

By sampling a range of species with different parity modes and degrees of placentotrophy in this study, we identify several key hypotheses surrounding the role of hormonal signaling from the chorioallantoic membrane in the evolution of pregnancy in amniotes. Several outstanding questions remain. Most importantly, it is not clear from our study where the signals sent from the chorioallantoic membrane are being received. Future work is necessary to identify if hormonal signals are being received by maternal tissues including the uterus in non-mammalian viviparous amniotes, or if the chorioallantoic membrane primarily acts to signal to the embryonic tissue and regulate development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2016.04.017.

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