Uterine Gene Expression in the Live-Bearing Lizard, *Chalcides ocellatus*, Reveals Convergence of Squamate Reptile and Mammalian Pregnancy Mechanisms

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Abstract

Although the morphological and physiological changes involved in pregnancy in live-bearing reptiles are well studied, the genetic mechanisms that underlie these changes are not known. We used the viviparous African Ocellated Skink, *Chalcides ocellatus*, as a model to identify a near complete gene expression profile associated with pregnancy using RNA-Seq analyses of uterine transcriptomes. Pregnancy in *C. ocellatus* is associated with upregulation of uterine genes involved with metabolism, cell proliferation and death, and cellular transport. Moreover, there are clear parallels between the genetic processes associated with pregnancy in mammals and *Chalcides* in expression of genes related to tissue remodeling, angiogenesis, immune system regulation, and nutrient provisioning to the embryo. In particular, the pregnant uterine transcriptome is dominated by expression of proteolytic enzymes that we speculate are involved both with remodeling the chorioallantoic placenta and histotrophy in the omphaloplacenta. Elements of the maternal innate immune system are downregulated in the pregnant uterus, indicating a potential mechanism to avoid rejection of the embryo. We found a downregulation of major histocompatability complex loci and estrogen and progesterone receptors in the pregnant uterus. This pattern is similar to mammals but cannot be explained by the mammalian model. The latter finding provides evidence that pregnancy is controlled by different endocrinological mechanisms in mammals and reptiles. Finally, 88% of the identified genes are expressed in both the pregnant and the nonpregnant uterus, and thus, morphological and physiological changes associated with *C. ocellatus* pregnancy are likely a result of regulation of genes continually expressed in the uterus rather than the initiation of expression of unique genes.

Key words: placenta, pregnancy, RNA-Seq, transcriptome, uterus, viviparity.

Introduction

Reproductive mode is subject to intense selective pressure and any modification will directly affect an organism's fitness. Thus, the transition between oviparity (egg-laying) and viviparity (live-bearing) is one of the most dramatic changes in the evolution of vertebrate animals. As a result of constraints on reproductive function, most vertebrate clades show a high phylogenetic conservatism in reproductive mode. For example, therian mammals represent only a single (albeit the best-known) origin of viviparity, whereas birds, alligators, and turtles exhibit only oviparity. By contrast, the evolutionary history of squamate reptiles (lizards and snakes) includes over 100 transitions from oviparity to viviparity, more than all other lineages of vertebrates combined (Blackburn 2006), with an extraordinary diversity of placental morphology and physiology (Blackburn et al. 1984; Blackburn 1993; Stewart 1993; Thompson and Speake 2006). Squamates are therefore an ideal model system with which to explore the processes that shape the evolution of viviparity and placentation in vertebrates.

Viviparity and placentation in squamate reptiles have been the subject of extensive morphological and physiological studies (e.g., Weekes 1935; Blackburn 1993; Thompson et al. 2000; Stewart and Thompson 2000; Thompson and Speake 2006). These studies have described a continuum of placental complexity, ranging from simple placentae formed by the apposition of uterine and embryonic tissue (e.g., most *Eulamprus quoyii* group skinks; Murphy et al. 2012) to highly complex placentae characterized by extensive folding of the uterus associated with blood vessels and containing enlarged uterine and chorionic epithelial cells (e.g., some *Mabuya* group skinks; Jerez and Ramírez-Pinilla 2001; Ramírez-Pinilla et al. 2006; Vieira et al. 2007; Blackburn and Flemming 2011). For the purposes of this discussion, we refer to these ends of the morphological complexity spectrum as "simple" and "complex," although we note these categories are an oversimplification of the true underlying diversity (see Blackburn 1993). Subsequent studies have quantified nutrient exchange across these different placental types and have concluded that very little maternal to embryonic transfer occurs via simple placentae, but substantial transfer occurs in species with more complex placentae. In one extreme, the embryos of the South American skink, *Mabuya bistrata* experience a 47,400% increase in dry mass (Vitt and Blackburn 1991) compared with an average of a 25% decrease in dry mass of oviparous species (Thompson et al. 2000).

Although much is known about the morphological and physiological changes involved in reptilian pregnancy, there has been little examination of the genetic architecture that underlies them (for review, see Murphy and Thompson 2011). Multiple studies have used a targeted gene approach to assess the expression of genes involved with remodeling of embryonic and maternal tissue (Biazik et al. 2007, 2008; Wu et al. 2011), increasing uterine blood supply to facilitate nutrient and gas exchange with the embryo (Murphy et al. 2009), modifying the maternal immune system to prevent rejection of embryos (Paulesu et al. 1995; Romagnoli et al. 2003), and supplying nutrients to the embryo in addition to those provided in the yolk (Biazik et al. 2009).

Although these studies provided important information into genes critical to maintaining squamate reptile pregnancy, the candidate gene approach used in those studies is not adequate to profile the potentially thousands of genes that are regulated during pregnancy in squamate reptiles. Indeed, until recently, it has not been technically feasible to investigate the genetic controls of viviparity and placental development in nonmammalian vertebrates because the necessary baseline genomic data have not been available. However, recent technological advances (RNA-Seq; Marioni et al. 2008) provide the first opportunity to both identify and quantify almost all of the genes expressed in the reptilian uterus. The integration of transcriptomic data with existing morphological and physiological data now allows us to elucidate the genetic mechanisms that underlie reptilian pregnancy, which are subject to drastic change during evolutionary transitions between reproductive modes.

Here, we conduct the first comprehensive examination of gene regulation in a pregnant and nonpregnant viviparous lizard uterus using RNA-Seq. Using the viviparous Ocellated Skink, *Chalcides ocellatus* as a model system, we provide a near complete gene expression profile associated with maintaining pregnancy (supplementary information 1, Supplementary Material online). *Chalcides ocellatus* is an ideal subject for this study because it possesses a relatively complex placenta with endometrial folds and histotrophic activity (Corso et al. 1988, 2000; it is roughly in the middle of the continuum of simple to very complex placenta) and because partial gene expression data exist on *Chalcides chalcides*, a congener used in previous candidate gene analyses (Paulesu et al. 1995, 2001; Romagnoli et al. 2003). We focus particularly on suites of genes associated with growth and remodeling of the uterus, the maternal immune system, transport across the placenta, and uterine metabolism because they are important functions of maintaining pregnancy and have also been the subject of mammalian, and to a lesser extent, squamate reptile gene expression analyses. We compare the gene expression profiles of the uterus of *C. ocellatus* with mammalian models to determine whether similar genetic mechanisms have evolved to maintain pregnancy mammals and reptiles. Similarities between reptiles and mammals in uterine gene expression would suggest convergent evolution of genetic mechanisms in response to similar natural selection pressures. On the other hand, significant differences in gene expression would be evidence that there are potentially many genetic pathways that permit the transition to viviparity (i.e., "many means to an end"). Both possibilities are inherently interesting because they allow us to interpret the general processes under which pregnancy has evolved in vertebrate animals.

Materials and Methods

Adult *C. ocellatus* individuals were purchased from a commercial dealer. Phylogenetic comparison of a 396-nt fragment of cytochrome b mitochondrial DNA (mtDNA) from these individuals with a comprehensive *Chalcides* mtDNA data set (Carranza et al. 2008) revealed them to be from Egyptian populations of *C. ocellatus ocellatus* (results not shown). The lizards were housed at Yale University in ~40 l glass aquaria with a 12 h light/dark photoperiod, a 24–37 °C thermal gradient (during the day), free access to water, and fed crickets three times a week. From a euthanized pregnant female, we dissected out a uterine egg chamber, removed the embryonic tissues attached to the uterus, and stored the uterus in RNA-Later (Ambion). The developmental state of the embryo was ~37 according to the normal development table of Defaure and Hubert

(1961). For a sample of nonpregnant uterus, we obtained an additional uterus from a female that was isolated for 6 weeks postpartum. There is no record of sperm retention in *C. ocellatus*, and the uterus showed no signs of pregnancy.

Total RNA was extracted from the uterus using an RNeasy Mini Kit (Qiagen). cDNA library construction and sequencing was performed by the Yale Center for Genome Analysis. The Illumina Genome Analyzer was used to sequence 72-bp pair-end fragments of cDNA libraries from both the pregnant and the nonpregnant samples.

There is no sequenced complete genome for *C. ocellatus*; indeed, only one complete genome of any nonavian reptile is currently sequenced (*Anolis carolinensis*), and it shares a common ancestor with *C. ocellatus* approximately 175 Ma (Brandley et al. 2008, 2011). To facilitate identification of sequenced transcripts, we therefore used ABySS 1.2.4 to assemble individual raw Illumina reads into larger contigs that are more easily identified. We used the subtractive multiple-*k* method of Surget-Groba and Montoya-Burgos (2010) and constructed multiple assemblies using a range of *k*-mer lengths (25, 29, 33, 37, 41, 45, 49, 53, 57, 61, 65, and 69). We then used CD-HIT-EST (Li and Godzik 2006) to remove redundant contigs and then removed contigs less than 100 bp (table 1).

To identify the contigs, we used BlastX with an *e* value of 10^{-5} to compare them with a database including 38 complete sequenced genomes of vertebrate animals (Ensembl build 61). We note that this methodology will not identify recently diverged paralogs and may therefore underestimate the true diversity of genes expressed in the uterus but that this is a potential problem for all transcriptomic studies and does not alter our broad conclusions. The raw Illumina data were filtered to remove any reads less than 30 bp. We then used the short-read aligner Bowtie 0.12.7 (Langmead et al. 2009) to align these filtered reads from both the nonpregnant and the pregnant uterine samples to a database composed of the identified contigs, allowing two nucleotide mismatches. Because of differing sequence quality between the forward and the reverse pair-end reads, we chose to only use the forward reads for alignment. Because both reads were sequenced from the same fragment, this should introduce little or no bias in the gene counts.

We assessed differential gene expression of normalized numbers of transcripts between the nonpregnant and the pregnant samples using the DEGseq (Wang et al. 2010) R package. Preliminary analysis revealed that some genes are massively upregulated in the pregnant uterus (e.g., 497,141 reads of cathepsin L1 in the pregnant uterus compared with 917 reads in the nonpregnant; see Results and Discussion). Because the number of cDNA fragments sequenced is finite, we were concerned that several highly expressed genes were sequenced at the expense of others, thereby artificially lowering the apparent expression of other genes (Robinson and Oshlack 2010). To try to mitigate this sequencing artifact, we used the trimmed mean of *M* values (TMM) method of Robinson and Oshlack (2010) in the edgeR R package (Robinson et al. 2010) to normalize the data and create an effective library size for both samples. We scaled both the pregnant and the nonpregnant libraries to "counts per 10^7 reads" using the following formula:

$$\text{Effective library size} = \frac{\text{Raw count of transcript} \times 10^7}{\text{Normalization factor} \times \text{Library size}},$$

where the TMM estimated normalization factor is 0.698231 for the pregnant library and 1.0 for the nonpregnant library and the original library sizes of 20,060,751 and 23,120,916, respectively. We then estimated differential expression of each gene between the nonpregnant and the pregnant samples using the MA plot–based method with random sampling model (MARS; Wang et al. 2010) in the DEGseq using a *P* value cutoff of 0.001 and a false discovery rate of 0.1%.

We used Gene Ontology (GO) analyses to broadly infer the biological function of the genes significantly regulated (as identified in the MARS differential expression analysis) in the uterine transcriptomes. We imported gene symbol lists of the significantly regulated genes in both the pregnant and the nonpregnant uteri into the Database for Annotation, Visualization, and Integrated Discovery (DAVID) functional GO annotation program and calculated GO terms corresponding to biological processes (BP-FAT). To more easily interpret the hundreds of individual GO biological functions inferred by DAVID, we aggregated similar processes to the following broad functional groups:

Catabolism—processes that breakdown complex molecules. This group contains any GO biological processes that were explicitly catabolic or proteolytic.

Cell death and differentiation—processes that regulate cellular proliferation, mitosis, and death.

Cell morphogenesis and development—processes involved with cell organization, including localization, cytoskeleton maintenance, tissue development, and motility.

Gene expression—processes that regulate transcription, translation, and posttranscriptional modification.

Homeostasis and response to stimuli—processes involved with maintaining homeostasis or reaction to stimuli.

Metabolism and energy—processes involved with the metabolism of macromolecules such as amino acids, lipids, nucleic acids, and proteins for the production of energy that are not explicitly catabolic (see above). This group also contains those mitochondrial processes involved in ATP production.

Molecule biosynthesis—processes that result in the production of macromolecules.

Signaling—processes involved with intracellular communication.

Table 1
Characteristics of Contigs Assembled by ABySS Using the Subtractive Multiple-*k* Method (Surget-Groba and Montoya-Burgos 2010)

Number of Contigs	**300,967**
Maximum contig length	**15,179**
Mean contig length	**530**
Median contig length	**165**
N50	**1,454**

Transport—processes that transport both organic and inorganic molecules both intracellularly and intercellularly.
Other—those processes that do not naturally fit into any of the above categories.

We grouped individual GO biological processes into the above categories provided they were represented by at least 50 unique genes. We note that these categories are not necessarily mutually exclusive as, for example, those processes involved in catabolism are also metabolic. However, we chose to identify separately explicit catabolic functions given the substantial upregulation of proteolytic and hydrolytic enzymes in the pregnant uterus (see Results and Discussion).

Results and Discussion

Our study is the first to survey the almost complete gene expression profile associated with maintaining pregnancy in a live-bearing reptile. Indeed, it is one of few studies that have used RNA-Seq to assess gene expression in any reptile (Gracheva et al. 2010; Schwartz et al. 2010; Wall et al. 2011). Our transcript identification analysis positively identified 12,070,543 of 20,060,751 (60.2%) transcripts in the pregnant and 12,330,621 of 23,120,916 (53.8%) transcripts in the nonpregnant *C. ocellatus* uterine transcriptome (supplementary information 1, Supplementary Material online). If we assume a minimum cutoff of $\geq$2 transcripts per million reads as our conservative criterion for calling a gene expressed, we identified 8,846 unique genes expressed in the pregnant and 8,881 unique genes expressed in the nonpregnant uteri, 8,296 (88%) of which are expressed in both the nonpregnant and the pregnant uterus. Our differential expression analysis using the MARS method in DEGseq identified a total of 3,903 upregulated and 2,709 downregulated genes in the pregnant *C. ocellatus* uterus, respectively. The 50 most upregulated and downregulated genes (as assessed by *z*-score) in the uterus of the pregnant *C. ocellatus* uterus, as well as specific genes discussed in the remainder of the paper, are provided in tables 2 and 3.

It is notable that many of the genes downregulated in the pregnant *C. ocellatus* uterus are genes involved with basic cellular housekeeping processes (e.g., ribosomal subunit proteins, desmin, myosin; table 3), which could indicate that the TMM normalization was inadequate and that these genes were undersampled. However, if these data were improperly normalized, then properly normalizing them would only increase the relative number of highly expressed transcripts in the pregnant uterus (table 2) and therefore does not severely impact our conclusions. We also emphasize that, given the lack of comparative genomic resources, we were unable to identify almost half of the sequenced transcripts and we have likely undersampled particularly quickly evolving genes—a challenge inherent in any RNA-Seq study of an organism with no closely related sequenced genome.

Most of the genes expressed in the pregnant uterus are also expressed in the nonpregnant uterus, which suggests that the morphological and physiological processes that maintain reptilian pregnancy are mostly a function of differential regulation of genes continually expressed in the uterus rather than the new expression of unique genes. If a similar pattern exists in the expression profiles of other oviparous and viviparous squamate reptiles, it would suggest that the evolutionary transition to viviparity is relatively "easy" given that the necessary genes are already expressed in the uterus when not gravid/pregnant and only become modulated in their expression level rather reprogrammed.

The GO biological function analysis revealed that *C. ocellatus* pregnancy is associated with upregulation of genes involved with catabolism, cell death and differentiation, homeostasis and response to stimuli, cellular signaling, and transport are upregulated in the pregnant uterus. By contrast, the nonpregnant uterus is dominated by genes that regulate cell morphogenesis and development, gene expression, and other processes.

Given the impracticality of examining the expression and function of each gene, we limit our discussion primarily to suites of genes that are associated with the biological functions that are apparently critical to maintaining pregnancy, including growth and remodeling of the uterus, nutrient transport across the placenta, and uterine metabolism and histotrophy. We also focus on the regulation of the maternal immune system and estrogen and progesterone activity, given their importance in maintaining mammalian pregnancy. We particularly focus on the most highly upregulated and downregulated genes identified by the MARS analysis and genes that have been assessed previously in squamate reptiles (table 4). Unless otherwise stated, we limit our discussion to only genes that are significantly upregulated or downregulated ($P \leq 0.001$) according to our MARS differential expression analysis. The entire results of the differential expression analysis for all genes are provided in the supplementary information 1 (Supplementary Material online).

Growth and Remodeling of the Uterus

The increasing demands of the growing embryo, including gas exchange, nutrition, and uterine space, require both fundamental destruction and growth of uterine tissue to both increase the size of the uterus and maximize the

Table 2
A Selection of Significantly Upregulated Genes in the Pregnant *Chalcides ocellatus* Uterus as Inferred by the MARS Differential Gene Expression Analysis of TMM Transformed Transcript Counts

Gene Symbol	Gene Name	Pregnant Transcript Count (Raw)	Nonpregnant Transcript Count (Raw)	Pregnant Transcript Count (Transformed)	Nonpregnant Transcript Count (Transformed)	Log_2-Fold Change	*Z*-Score	Expression Rank
ACTB	Actin, beta	95,506	39,786	133,776	55,728	1.3	154.2	16
AGTRAP	Angiotensin II receptor–associated protein	1,177	657	840	284	1.6	16.9	1,058
ALDOA	Aldolase A	10,459	9,017	7,467	3,900	0.9	33.7	384
ALDOB	Aldolase B	7,194	291	5,136	126	5.3	74.4	94
ALDOC	Aldolase C	275	123	196	53	1.9	9.3	1,991
ANG	Angiogenin	14,189	4,337	10,130	1,876	2.4	78.7	80
ANGPT4	Angiopoietin 4	451	199	322	86	1.9	12.0	1,575
ANXA4	Annexin A4	12,674	3,524	9,048	1,524	2.6	76.8	87
APOA1	Apolipoprotein A-I	102	2	73	1	6.2	8.9	2,090
APOA2	Apolipoprotein A-II	1,123	0	802	0	10.6	23.4	720
APOA4	Apolipoprotein A-IV	331	109	236	47	2.3	11.7	1,617
APOE	Apolipoprotein E	8,568	2,879	6,117	1,245	2.3	59.1	145
APOM	Apolipoprotein M	34	2	24	1	4.6	5.0	3,188
AQPP11	Aquaporin 11	123	28	88	12	2.9	8.0	2,279
AQPP3	Aquaporin 3	859	837	613	362	0.8	8.1	2,266
AQPP5	Aquaporin 5	2,391	18	1,707	8	7.7	41.0	279
ARG2	Amino acid acetyltransferase	16,563	81	23,200	113	7.7	128.0	22
ATP1A1	ATPase, Na^+/K^+ transporting, alpha 1	54,438	10,240	38,865	4,429	3.1	176.0	11
BPI2	Bactericidal/permeability-increasing protein 2	17,327	97	12,370	42	8.2	107.9	40
BTG1	B-cell translocation gene 1	16,577	1,535	11,835	664	4.2	107.9	41
CA2	Carbonic anhydrase II	7,873	749	5,621	324	4.1	74.1	95
CAT	Catalase	41,302	604	57,852	846	6.1	211.2	8
CDH1	Cadherin 1	12,711	3,753	9,075	1,623	2.5	75.4	91
CDH11	Cadherin 11	20,807	634	14,855	274	5.8	126.8	24
CDH15	Cadherin 15	1,735	1,214	1,239	525	1.2	17.2	1,037
CDH2	Cadherin 2	685	717	489	310	0.7	6.4	2,732
CDH24	Protocadherin 24	99	60	71	26	1.4	4.7	3,305
CDH4	Cadherin 4	4,170	21	2,977	9	8.4	52.5	185
CHY	Chymosin	12,199	12	8,709	5	10.8	76.2	88
CKB	Creatine kinase	20,847	3,963	14,883	1,714	3.1	108.6	38
CLDN1	Claudin 1	9,577	601	6,837	260	4.7	84.4	67
CLDN12	Claudin 12	214	222	153	96	0.7	3.6	3,732
CLDN23	Claudin 23	92	60	66	26	1.3	4.2	3,478
CLDN3	Claudin 3	2,806	2,719	2,003	1,176	0.8	14.8	1,245
CLDN4	Claudin 4	8,586	4,014	6,130	1,736	1.8	51.0	197
CLDN5	Claudin 5	557	587	398	254	0.6	5.7	2,963
CLDN6	Claudin 6	3,666	2,451	2,617	1,060	1.3	26.1	613
CLDN7	Claudin 7	3,596	1,716	2,567	742	1.8	32.6	417
CLDN8	Claudin 8	5,015	111	3,580	48	6.2	62.1	135
CO3	Cytochrome c oxidase subunit 3	37,325	14,477	52,281	20,278	1.4	102.2	48
COL6A3	Collagen, type VI, alpha 3	14,847	1,170	20,796	1,639	3.7	116.0	28
CPA2	Carboxypeptidase A2	18,052	4,020	25,286	5,631	2.2	98.1	50
CSTC	Cystatin C	18,365	1,827	13,111	790	4.1	112.7	35
CSTM	Cystatin 6/M/E	30,367	95	21,680	41	9.0	136.2	20
CTSA	Cathepsin A	6,942	1,022	4,956	442	3.5	65.8	119
CTSB	Cathepsin B	17,222	5,309	12,295	2,296	2.4	86.5	64
CTSD	Cathepsin D	10,474	7,033	7,478	3,042	1.3	43.9	253
CTSE	Cathepsin E	67	7	48	3	4.0	6.8	2,630
CTSF	Cathepsin F	1,269	1,105	906	478	0.9	11.6	1,633
CTSL	Cathepsin L1	497,141	918	354,922	397	9.8	523.9	1

Table 2
Continued

Gene Symbol	Gene Name	Pregnant Transcript Count (Raw)	Nonpregnant Transcript Count (Raw)	Pregnant Transcript Count (Transformed)	Nonpregnant Transcript Count (Transformed)	Log_2-Fold Change	*Z*-Score	Expression Rank
CTSV	Cathepsin V (cathepsin L2)	106,855	129	76,287	56	10.4	232.0	6
CTSZ	Cathepsin Z	65,117	999	46,489	432	6.7	221.5	7
CYB	Cytochrome b	39,111	14,696	54,783	20,585	1.4	107.3	44
DDIT4	DNA damage–inducible transcript 4	15,069	569	10,758	246	5.5	107.8	42
DSG2	Desmoglein 2	2,002	946	1,429	409	1.8	24.5	676
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2	13,373	60	9,547	26	8.5	93.2	56
EPAS1	Endothelial PAS domain protein 1	41,345	2,946	29,517	1,274	4.5	174.1	12
FABP1	Fatty acid–binding protein 1	14,898	0	10,636	0	14.4	60.2	140
FABP2	Fatty acid–binding protein 2	97	2	69	1	6.1	8.6	2,147
FABP3	Fatty acid–binding protein 3	2,247	395	1,604	171	3.2	36.2	340
FABP4	Fatty acid–binding protein 4	3,051	523	2,178	226	3.3	42.4	268
FABP5	Fatty acid–binding protein 5	1,198	388	855	168	2.3	22.4	765
FABP7	Fatty acid–binding protein 7	12,174	2	8,691	1	13.1	61.9	136
FN1	Fibronectin protein	15,382	2,046	21,546	2,866	2.9	106.9	45
FTL	Ferritin, light polypeptide	36,692	10,208	51,395	14,298	1.8	126.0	25
FUCA1	Fucosidase, alpha-L1	145,697	220	104,017	95	10.1	277.5	5
FUCA2	Fucosidase, alpha-L2	129,789	765	92,660	331	8.1	296.6	4
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	29,268	15,408	20,895	6,664	1.6	87.8	62
GGH	Gamma-glutamyl hydrolase	12,615	85	17,670	119	7.2	113.8	33
GLA	Galactosidase, alpha	16,970	486	12,115	210	5.9	114.5	32
GLB1	Galactosidase, beta 1	14,934	513	10,662	222	5.6	107.4	43
GLUL	Glutamate–ammonia ligase (glutamine synthetase)	50,207	3,729	35,844	1613	4.5	191.3	10
GRN	Granulin	10,060	1,385	7,182	599	3.6	80.0	74
GSN	Gelsolin	19,199	5,489	13,707	2,374	2.5	93.7	54
GPX1	Glutathione peroxidase 1	3,871	1,307	5,422	1,831	1.6	36.4	337
GPX3	Glutathione peroxidase 3	2,969	23	4,159	32	7.0	55.6	161
GPX4	Glutathione peroxidase 4	2,996	957	4,197	1,340	1.6	33.2	396
GPX5	Glutathione peroxidase 5	1,484	22	2,079	31	6.1	40.0	287
HEXA	Hexosaminidase A	196,426	183	140,234	79	10.8	305.3	3
HEXB	Hexosaminidase B	15,870	192	11,330	83	7.1	108.3	39
HMGCS2	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 2	9,362	55	6,684	24	8.1	79.7	76
HYAL3	Hyaluronoglucosaminidase 1	17,923	46	12,796	20	9.3	102.8	47
HYAL4	Hyaluronoglucosaminidase 3	12,479	0	8,909	0	14.1	56.5	157
IFI30	Interferon gamma–inducible protein 30/GILT	11,020	138	15,436	193	6.3	108.8	37
IL20RB	Interleukin 20 receptor beta	8,497	261	6,066	113	5.7	81.0	69
KRT18	Keratin 18	19,527	3,222	27,352	4,513	2.6	113.5	34
KRT8	Keratin 8	20,650	3,619	28,925	5,069	2.5	114.5	31
LDHA	Lactate dehydrogenase A	10,376	1,447	7,408	626	3.6	81.1	68
LGMN	Legumain	57,922	430	41,352	186	7.8	201.3	9
MDH1	Malate dehydrogenase 1	8,071	3,725	5,762	1,611	1.8	49.7	207
MDH2	Malate dehydrogenase 2	5,076	3,831	3,624	1,657	1.1	27.4	572
NAGA	Alpha-*N*-acetylgalactosaminidase	25,652	354	18,314	153	6.9	138.4	19
NDRG1	*N*-myc downstream–regulated 1	26,922	1,595	19,220	690	4.8	142.0	18
NEU1	Sialidase 1	29,234	76	20,871	33	9.3	131.4	21
NFAT5	Nuclear factor of activated T-cells 5	10,795	785	15,121	1,100	3.8	100.0	49
OVGP1	Oviductal glycoprotein 1, 120 kDa	29,231	217	40,944	304	7.1	174.0	13
PCK2	Phosphoenolpyruvate carboxykinase 2	8,634	511	6,164	221	4.8	80.4	73
PGAM1	Phosphoglycerate mutase 1	11,543	2,573	8,241	1,113	2.9	77.9	83
PKM2	Pyruvate kinase, isozymes M1/M2	25,858	6,134	18,461	2,653	2.8	114.8	30

Table 2
Continued

Gene Symbol	Gene Name	Pregnant Transcript Count (Raw)	Nonpregnant Transcript Count (Raw)	Pregnant Transcript Count (Transformed)	Nonpregnant Transcript Count (Transformed)	Log_2-Fold Change	*Z*-Score	Expression Rank
PLA2G1B	Phospholipase A2, group IB	22,787	187	16,268	81	7.6	127.0	23
PPIB	Peptidyl-prolyl *cis-trans*-isomerase B	16,423	2,048	23,004	2,869	3.0	112.1	36
PRCP	Prolylcarboxypeptidase (angiotensinase C)	2,944	173	2,102	75	4.8	47.0	229
PRSS1	Protease, serine, 1	2,681	0	1,914	0	11.9	32.4	420
PRSS12	Protease, serine, 12	1,290	49	921	21	5.5	31.5	443
PRSS16	Protease, serine, 16	90,516	9	64,622	4	14.0	154.5	15
PRSS2	Protease, serine, 2	148,046	2	105,694	1	16.7	147.5	17
PRSS27	Protease, serine, 27	19,852	1,096	14,173	474	4.9	122.3	26
PRSS3	Protease, serine, 3	161,271	5	115,136	2	15.8	169.8	14
PRSS33	Protease, serine, 33	994	60	710	26	4.8	27.3	578
PRSS36	Protease, serine, 36	466	12	333	5	6.1	19.0	935
PRSS8	Protease, serine, 8	11,814	400	8,434	173	5.6	95.5	52
REN	Renin	15,355	32	10,962	14	9.6	93.3	55
SLC15A1	Solute carrier family 15, member 1	12,659	22	17,732	31	9.2	103.2	46
SPINT1	Serine peptidase inhibitor, Kunitz type 1	10,400	1,020	7,425	441	4.1	84.9	66
SPINT2	Serine protease inhibitor, Kunitz type 2	14,494	4,573	10,348	1,978	2.4	78.7	81
TFPI2	Tissue factor pathway inhibitor 2	17,908	393	12,785	170	6.2	117.3	27
TPP1	Polynucleotide 3′-phosphatase	162,913	2,062	116,308	892	7.0	347.8	2
VEGFA	Vascular endothelial growth factor A	3,348	1,727	2,390	747	1.7	30.1	495
WSB1	WD repeat and SOCS box–containing 1	14,527	1,088	20,348	1,524	3.7	115.6	29

NOTE.—Only the top 50 most upregulated genes (as assessed by *z*-score), and those genes discussed in the text, are shown. The expression rank represents the order of expression in the entire upregulated transcriptome from highest to lowest (e.g., a rank of 1 is the highest change in expression). Complete results for the MARS analysis are provided in supplementary information 1 (Supplementary Material online).

surface area of the maternal–embryonic interface. Genes likely involved in these remodeling process are some of the most highly upregulated in the pregnant *C. ocellatus* uterus (table 2) and include cytoskeletal elements (e.g., β-actin and keratins) and gelsolin (a powerful actin regulator), genes that promote (e.g., N-myc downstream regulated 1, Granulin) and inhibit (e.g., B-cell translocation gene 1, DNA damage–inducible transcript four) cell proliferation, and hyaluronoglucosaminidases 3 and 4, that break down the extracellular matrix.

Although there are potentially hundreds or thousands of genes involved in the uterine remodeling process, we focus on the proteolytic enzymes because they are massively upregulated and have been the subjects of multiple studies of mammalian pregnancy. We also evaluate expression of the highly upregulated cell adhesion and angiogenic genes because they have been subject to previous studies in squamate reptiles (Biazik et al. 2007, 2008, 2010; Murphy, Belov, et al. 2010; Wu et al. 2011) and therefore provide a rare opportunity to assess the diversity of molecular mechanisms involved in reptilian pregnancy.

Proteases

Because the close apposition of maternal and fetal tissue facilitates gas and nutrient exchange, uterine and placental tissues are remodeled to minimize this distance, regardless of whether the placental type is epitheliochorial, endotheliachorial, or hemochorial (Enders and Carter 2004). The destruction of this tissue is achieved, in part, via the actions of both cysteine and serine proteases (Salamonsen and Nie 2002). Perhaps the most striking gene expression upregulation in the pregnant *C. ocellatus* uterine transcriptome is the enormous upregulation of cysteine and serine proteases. Cathepsins (CTS) are lysosomal cysteine proteases that degrade extracellular matrix and catabolize intracellular proteins (Kirschke et al. 1997). Cathepsins make up over 8% of the entire upregulated transcriptome, with CTSL alone representing 5% of the transcriptome. The serine proteases PRSS2 (trypsin) and PRSS3 (mesotrypsin) have the highest log_2-fold changes of all the upregulated genes (16.7 and 15.5, respectively; table 2). Taken together with other highly upregulated proteases such as legumain (LGMN) and tripeptidyl peptidase I (TPP1), these results reveal that the breakdown of tissue is one of the most active physiological processes in the *C. ocellatus* pregnant uterus.

Although the roles of cathepsins, especially CTSB and CTSL, in uterine remodeling has been elucidated in several mammal species (e.g., Afonso et al. 1997; Divya et al. 2002; Song et al. 2006, 2010; Varanou et al. 2006), comparison of cathepsin expression between *C. ocellatus* lizards and pigs is

Table 3
A Selection of Significantly Downregulated Genes in the Pregnant *Chalcides ocellatus* Uterus as Inferred by the MARS Differential Gene Expression Analysis of TMM Transformed Transcript Counts

Gene Symbol	Gene Name	Pregnant Transcript Count (Raw)	Nonpregnant Transcript Count (Raw)	Pregnant Transcript Count (Transformed)	Nonpregnant Transcript Count (Transformed)	Log_2-Fold Change	*Z*-Score	Expression Rank
ACTA2	Actin, alpha 2	1,340	7,007	1,877	16,201	−2.4	−64.8	19
AHNAK	AHNAK nucleoprotein	6,465	15,345	9,056	35,479	−1.2	−61.0	23
BTF3	Basic transcription factor 3	3,393	9,969	4,753	23,049	−1.6	−58.1	28
C3	Complement component 3	2,256	10,334	3,160	23,893	−2.2	−74.8	9
CALD1	Caldesmon 1	9,97	5,162	1,397	11,935	−2.4	−55.4	31
CD74	CD74 molecule	503	4,806	705	11,112	−3.3	−62.9	21
CDH13	Cadherin 13	48	105	67	243	−1.1	−4.7	2164
CDH5	Cadherin 5	40	246	56	569	−2.6	−12.8	686
CNN1	Calponin 1	612	4,810	857	11,121	−3.0	−60.4	25
COL12A1	Collagen, type XII, alpha 1	306	6,613	429	15,290	−4.4	−82.0	6
CXCL14	Chemokine (C-X-C motif) ligand 14	56	3,551	78	8,210	−6.0	−62.0	22
DCN	Decorin	361	3,191	506	7,378	−3.1	−50.5	41
DDX50	DEAD (Asp-Glu-Ala-Asp) box polypeptide 50	8,260	23,789	11,570	55,002	−1.5	−88.6	4
DES	Desmin	1,883	9,420	2,638	21,780	−2.3	−73.9	11
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	448	3,065	628	7,087	−2.8	−46.5	48
ESR1	Estrogen receptor 1	499	7,745	699	17,907	−4.0	−86.0	5
GAS1	1,3-Beta-glucanosyltransferase GAS1	1304	4,980	1,827	11,514	−1.9	−47.8	42
GPI	Glucose-6-phosphate isomerase	6,118	14,803	8,570	34,226	−1.3	−61.0	24
GPR124	G protein–coupled receptor 124	228	4,267	319	9,866	−4.2	−65.1	18
HMGB1	High-mobility group 1	856	4,490	1,199	10,381	−2.4	−51.9	39
HSP90AB1	Heat shock protein 90 kDa alpha, class B member 1	6,564	15,181	9,194	35,100	−1.2	−59.3	27
IFITM3	Interferon-induced transmembrane protein 3	568	3,435	796	7,942	−2.6	−47.6	44
IGFBP5	Insulin-like growth factor–binding protein 5	289	5,002	405	11,565	−4.1	−69.9	12
LGR5	Leucine-rich repeat-containing G protein–coupled receptor 5	436	8,664	611	20,032	−4.3	−93.2	1
MEIS1	Meis homeobox 1	316	2,680	443	6,196	−3.1	−45.9	49
MHCIA	MHC Class IA	1,651	11,834	2,313	27,361	−2.8	−92.6	2
MHCIIA	MHC Class IIA	637	5,550	892	12,832	−3.1	−66.4	17
MHCIIB	MHC Class IIB	835	4,703	1,170	10,874	−2.5	−54.4	34
MYH11	Myosin, heavy chain 11	1,940	9,598	2,717	22,191	−2.3	−74.3	10
PCLP1	Podocalyxin-like protein 1	965	6,741	1,352	15,586	−2.8	−69.4	13
PDLIM4	PDZ and LIM domain 4	364	6,474	510	14,968	−4.2	−79.8	8
PGR	Progesterone receptor	283	2,345	396	5,422	−3.1	−42.7	61
PTGIS	Prostaglandin I2 (prostacyclin) synthase	131	1,580	183	3,653	−3.6	−37.6	93
RPL10A	60S ribosomal protein L10A	5,856	17,979	8,203	41,569	−1.6	−80.4	7
RPL14	50S ribosomal protein L14	3,239	8,857	4,537	20,478	−1.5	−52.1	38
RPL17	50S ribosomal protein L17	6,268	13,974	8,780	32,309	−1.2	−54.9	32
RPL18A	50S ribosomal protein L18A	2,656	10,063	3,720	23,267	−1.9	−67.7	16
RPL19	50S ribosomal protein L19	4,645	10,270	6,506	23,745	−1.1	−46.6	47
RPL27A	50S ribosomal protein L27A	2,685	8,070	3,761	18,659	−1.6	−53.1	35
RPL32	60S ribosomal protein L32	3,513	8,440	4,921	19,514	−1.3	−45.7	50
RPL37	60S ribosomal protein L37A	2,163	8,028	3,030	18,561	−1.9	−59.9	26
RPL4	60S ribosomal protein L4	8,509	19,769	11,919	45,708	−1.2	−67.9	15
RPLP2	60S ribosomal protein LP2	2,023	6,650	2,834	15,375	−1.7	−51.0	40
RPS13	40S ribosomal protein S13	3,213	9,151	4,500	21,158	−1.5	−54.5	33
RPS2	40S ribosomal protein S2	12,945	22,719	18,132	52,528	−0.8	−52.1	37
RPS23	40S ribosomal protein S23	2,612	7,930	3,659	18,335	−1.6	−53.0	36

Table 3
Continued

Gene Symbol	Gene Name	Pregnant Transcript Count (Raw)	Nonpregnant Transcript Count (Raw)	Pregnant Transcript Count (Transformed)	Nonpregnant Transcript Count (Transformed)	Log_2-Fold Change	*Z*-Score	Expression Rank
RPS7	40S ribosomal protein S7	2,504	13,326	3,507	30,811	−2.4	−89.9	3
RPS8	40S ribosomal protein S8	3,464	11,054	4,852	25,558	−1.7	−64.6	20
RPS9	40S ribosomal protein S9	5,272	11,266	7,385	26,048	−1.1	−47.2	45
SLC6A6	Solute carrier family 6, member A6	845	4,998	1,184	11,556	−2.6	−57.0	29
SLIT2	Slit homolog 2	276	2,727	387	6,305	−3.3	−47.7	43
SOX7	SRY (sex-determining region Y)-box 7	318	2,784	445	6,437	−3.1	−47.1	46
TNS3	Tensin 3	675	6,013	945	13,903	−3.2	−69.4	14
TPBG	Trophoblast glycoprotein	271	3,464	380	8,009	−3.7	−56.1	30

NOTE.—Only the top 50 most downregulated genes (as assessed by *z*-score), and those genes discussed in the text, are shown. The expression rank represents the order of expression in the entire nonpregnant uterine transcriptome from highest to lowest (e.g., a rank of 1 is the highest change in expression). Complete results for the MARS analysis are provided in supplementary information 1 (Supplementary Material online).

most instructive because both possess an epitheliochorial placenta. In pigs, CTSB and CTSL are expressed in both uterine and chorionic epithelia, and this expression increases as pregnancy progresses (Song et al. 2010). Both CTSB and CTSL are upregulated in the pregnant *C. ocellatus* uterus, CTSL being the most abundant transcript in the transcriptome. Although we cannot localize expression of these genes to a specific region of the uterus, we nonetheless speculate that, as in mammals, these genes may have a primary role in degrading uterine tissue during the remodeling process in the *C. ocellatus* uterus.

Placental remodeling is an interplay between proteases and their inhibitors (Afonso et al. 1997; Salamonsen and Nie 2002; Song et al. 2006, 2007, 2010), especially the reciprocal actions of cathepsins and their inhibitors, cystatins (CST). Cystatin C (CSTC) is a powerful inhibitor of cathepsins L and B and is highly upregulated in *C. ocellatus*. CTSL and two other lysosomal cysteine proteases, CTSV (also called CTSL2) and LGMN, are also inhibited by cystatin M/E (also called cystatin 6), a very highly upregulated gene in the pregnant *C. ocellatus* transcriptome. Moreover, multiple serine protease inhibitors, including tissue factor pathway inhibitor 2 (TFPI2), serine peptidase inhibitor, Kunitz types 1 and 2 (SPINT1 and 2), are significantly upregulated, thereby suggesting active regulation of serine protease activity. Thus, there is a clear pattern of protease expression and concomitant regulation by their inhibitors. Moreover, the genes likely responsible for these processes are identical to those used by mammals.

Cell Adhesion

Remodeling of the uterus throughout pregnancy requires the growth and movement of the closely apposed maternal and embryonic surface epithelial cells and presumably the molecules that permit adhesion of these cells to one another. These cellular adhesion proteins, including those critical to the development of desmosomes, tight junctions, and adherens junctions, are some of the few proteins extensively studied in squamate reptiles (Australian skinks; Biazik et al. 2007, 2008, 2010; Wu et al. 2011) and therefore provide a rare opportunity to assess gene expression differences amongst multiple species of viviparous reptiles (table 4).

Desmoglelin 2 (DSG2) is a protein involved in the formation of desmosomes and its expression has been evaluated in the uteri of four scincid lizards representing the extremes of placental development (Biazik et al. 2010). DSG2 expression is not different in pregnant and nonpregnant females in two species with simple placentae (*Lerista bougainvillii* and *Saiphos equalis*) but is lower during pregnancy in two species with complex placentae (*Pseudemoia entrecasteauxii* and *Pseudemoia spenceri*), which suggests that a decrease in desmosome number may allow for easier deformation and remodeling of the uterus. In contrast, our transcriptome results demonstrate a statistically significant increase in DSG2 expression in the uterus of pregnant *C. ocellatus* and in the absence of corroborating morphological data, presumably an increasing number of desmosomes.

In addition to simple cell adhesion, tight junctions regulate paracellular transport by controlling the permeability of the interstitial space between adjacent cells to molecules of specific sizes. Occludin (OCLN) and claudins (CLDN) are the dominant proteins in tight junctions. OCLN expression increases as pregnancy progresses in the uteri of two viviparous species of skinks with complex placentation (*P. entrecasteauxii* and *P. spenceri*), suggesting decreased paracellular permeability with compensation by transcellular transport (Biazik et al. 2007). OCLN expression was not detected in two species with simple placentae (*L. bougainvillii* and *S. equalis*; Biazik et al. 2007). Similarly, our transcriptomic analysis finds no significant difference in OCLN expression between nonpregnant and pregnant *C. ocellatus* females ($P = 0.187$), although this species has a more complex placenta. CLDN5 is expressed over the entire surface of the uterine epithelial cell in early pregnancy in four

Table 4
Genes Whose Regulation Has Been Assessed Previously in Squamate Reptiles and Comparison with *Chalcides ocellatus*

Gene	Species	Regulatory Activity during Pregnancy	Reference
Desmoglein 2 (DSG2)	*Chalcides ocellatus*	Increase	This study
	Lerista bougainvillii[a]	No change	Biazik et al. (2010)
	Pseudemoia entrecasteauxii	Decrease	Biazik et al. (2010)
	Pseudemoia spenceri	Decrease	Biazik et al. (2010)
	Saiphos equalis[a]	No change	Biazik et al. (2010)
Occludin (OCLN)	*C. ocellatus*	No change	This study
	Eulamprus tympanum	Absent	Biazik et al. (2007)
	P. entrecasteauxii	Absent	Biazik et al. (2007)
	P. spenceri	Increase	Biazik et al. (2007)
	S. equalis[a]	Increase	Biazik et al. (2007)
Claudin 5 (CLDN5)	*C. ocellatus*	Increase	This study
	E. tympanum	Present	Biazik et al. (2008)
	L. bougainvillii[a]	Present	Biazik et al. (2008)
	P. entrecasteauxii	Present	Biazik et al. (2008)
	P. spenceri	Present	Biazik et al. (2008)
	S. equalis[a]	Present	Biazik et al. (2008)
Cadherin (CDH)[b]	*C. ocellatus*	Varied, but mostly increase	This study
	L. bougainvillii[a]	Decrease	Wu et al. (2011)
	Niveoscincus metallicus	Decrease	Wu et al. (2011)
	Niveoscincus ocellatus	Decrease	Wu et al. (2011)
Vascular endothelial growth factor A (VEGFA)	*C. ocellatus*	Increase	This study
	E. tympanum	Present	Murphy, Belov, et al. (2010)
	N. metallicus	Present	Murphy, Belov, et al. (2010)
	P. entrecasteauxii	Present	Murphy, Belov, et al. (2010)
	S. equalis[a]	Increase	Murphy, Belov, et al. (2010)
Interleukin 1A (IL1A)	*C. ocellatus*	Absent	This study
	Chalcides chalcides	Present	Paulesu et al. (1995)
	Zootoca (Lacerta) vivipara[a]	Present	Paulesu et al. (2005)
Interleukin 1B (IL1B)	*C. ocellatus*	Absent	This study
	C. chalcides	Present	Paulesu et al. (1995)
	Z. vivipara[b]	Present	Paulesu et al. (2005)

NOTE.—Gene expression is identified as "present" if expression was inferred, but levels were not quantified between pregnant and nonpregnant individuals. Gene expression is identified as "no change" if expression was quantified, but there is no change in expression between pregnant and nonpregnant individuals.

[a] Reproductively bimodal species. Only data for the viviparous individuals are presented.

[b] Nonspecific "pan-cadherin" antibody used.

viviparous skink species but becomes localized at the tight junctions, suggesting an increased role of the tight junctions in limiting paracellular transport (Biazik et al. 2008). As no study has examined tight junction distribution in *C. ocellatus*, we cannot directly compare our results with Biazik et al. (2008), but we do find significant upregulation of nine claudin loci, including CLDN1, 3–8, 12, and 23, suggesting that they have an important role in maintaining pregnancy in *C. ocellatus*.

Cadherins (CDH) are a family of proteins that are components of adherens junctions between adjacent cells. Cadherin expression is high near ovulation, as revealed using an antibody that labels all cadherins but significantly decreases as pregnancy progresses and is absent in late pregnancy in three species of Australian skinks (Wu et al. 2011). Thus, decreasing cadherin expression, and therefore few adherens junctions, is speculated to allow the uterus to expand to accommodate the growing embryo (Wu et al. 2011). In *C. ocellatus*, we detected a far more complicated expression profile of cadherins with significant upregulation of CDH1 (epithelial cadherin), 2, 4, 11, 15, 22, and 24 and downregulation of CDH5 and 13. This indicates that, unlike the three Australian skinks studied to date, adherens junctions actually proliferate in *C. ocellatus* during pregnancy and apparently do not inhibit uterine growth.

Our transcriptomic analyses reveal some significant differences between our study and previous studies of cell adhesion molecules in the uteri of viviparous skinks. These differing results could be due to the higher resolution of gene expression possible with transcript sequencing as opposed to immunological assays or western blots used in previous studies (Biazik et al. 2007, 2008, 2010; Wu et al. 2011). However, rather than any methodological problem, a much more plausible explanation for the difference in

uterine cell adhesion gene expression is that the study organisms represent ancient independent derivations of viviparity. Although also a skink, *C. ocellatus* and Australian lygosomine skinks share a common ancestor approximately 95 Ma (Brandley et al. 2008, 2011), about the same age as the common ancestor of primates, ungulates, carnivores, and bats (Murphy et al. 2007). It is therefore reasonable to hypothesize that these two skink lineages evolved different molecular processes and suggests that there is a diversity of genetic processes that act to maintain pregnancy within viviparous squamate reptiles.

Angiogenesis and Vascular Physiology

As the embryo matures, its growing oxygen demand is accommodated by increasing placental vascularization (Guillette and Jones 1985; Masson and Guillette 1987; Murphy, Belov, et al. 2010; Murphy, Parker, et al. 2010; Parker, Manconi, et al. 2010; Parker, Murphy, et al. 2010). Like mammals, increasing expression of vascular endothelial growth factor A (VEGFA) in pregnant squamate reptiles is correlated with vascular growth in the uterus (Murphy, Belov, et al. 2010). VEGFA is also significantly upregulated in the uterus of pregnant *C. ocellatus*. VEGFA is upregulated in addition to a suite of other genes with angiogenic properties. Most notable are the endothelial PAS domain protein 1 (EPAS1) and hypoxia-inducible factor 1α (HIF1A) genes that activate VEGF expression in response to hypoxic conditions (Forsythe et al. 1996; Peng et al. 2000; Takeda et al. 2004); interleukin 20 receptor β (IL20RB), an inducer of endothelial cell proliferation (Gaspar et al. 2002; Hsieh et al. 2006); angiogenin (ANG) and angiopoietin 4 (ANGPT4), and general cell proliferation genes with angiogenic properties such as ENPP2/Autotaxin. As in remodeling the structure of the uterus, proteolytic enzymes such as cathepsins are also associated with angiogenesis (Joyce et al. 2004).

In addition to genes that promote the development of blood vessels, several genes that control blood physiology are highly expressed in the *C. ocellatus* pregnant uterus. Prostacyclin (PTGIS), a vasodilator, is downregulated. Renin, a part of the renin–angiotensin system (RAS) that regulates blood pressure, is highly upregulated suggesting intense vasoconstriction of the uterine vasculature and consequently high blood pressure (perhaps facilitating the diffusion of oxygen and nutrients to the embryo across the placenta). Renin is expressed in the uteroplacental tissues of mammals where it participates in a local RAS (Cooper et al. 1999; Nielsen et al. 2000; Vinson et al. 1997) that may also stimulate cell proliferation and angiogenesis (Hagemann et al. 1994). However, renin does not directly increase blood pressure but rather converts angiotensinogen (AGT) to angiotensin I, which is then converted to the much more potent vasoconstrictor angiotensinogen II via action of angiotesin-converting enzyme (ACE). Angiotensinogen (AGT) is not significantly regulated in the pregnant *C. ocellatus* uterus, and we found no expression of ACE. Moreover, prolylcarboxypeptidase/angiotensinase C (PRCP) and angiotensin II receptor–associated protein (AGTRAP), two inhibitors of angiotensin II, are upregulated and therefore suggest that blood pressure is locally regulated by the expression of vasoconstrictors and dilators.

Regulation of the Maternal Innate Immune System

The embryo is an allograft of both maternal and paternal tissue and is therefore at risk of attack by the maternal immune system. As such, a variety of mechanisms that "hide" the embryo have evolved in mammals by downregulating elements of the maternal immune system (Moffett and Loke 2006). This immune response is expected to be much higher in mammals with invasive placentation because the maternal epithelia are breached. With a single possible exception (*Trachylepis* [*Mabuya*] *ivensii*; Blackburn and Flemming 2011), all viviparous squamate reptiles studied to date, including *C. ocellatus*, possess an epitheliochorial placenta with no breaching of the maternal epithelia. Nonetheless, as pregnancy progresses in squamate reptiles, the remnants of the ancestral shell membrane commonly disintegrate, thereby creating direct contact between the embryonic and the maternal epithelia (Blackburn 1993). Although the epithelia remain intact, studies of the mammalian epitheliochorial placentae demonstrate adaptations to hide from the maternal immune system (Moffett and Loke 2006) by regulating the maternal innate immune system and embryonic major histocompatability (MHC) loci.

The innate immune system comprises the general mechanisms by which the body defends against infection in a nonspecific manner. Its components consist of leukocytes (e.g., macrophages, natural killer cells), general antimicrobial proteins (e.g., bactericidal/permeability-increasing protein 2), and molecules that promote inflammation (e.g., cytokines, chemokines). It differs from the adaptive immune system that recognizes and remembers specific pathogens and thereby provides long-term immunity. Inflammation is an innate immune response critical to fighting infection, but excessive uterine inflammation may also lead to spontaneous abortion (Challis et al. 2009). Therefore, regulation of the proinflammatory and anti-inflammatory molecules in the pregnant uterus is essential to the maintenance of pregnancy while maintaining the maternal immune response to pathogens (Walker et al. 2010). Inflammation is regulated by a suite of genes, many of them cytokines—molecules that initiate cascades of intracellular signaling that are particularly active in the innate immune system. In squamate reptiles, the interleukin 1 cytokine system has received particular attention. IL1A and IL1B, two proinflammatory cytokines that also initiate immune response and regulate cellular growth and differentiation, are expressed in the uterine epithelia of *C. chalcides*, a close relative of *C. ocellatus*, and this expression continues throughout pregnancy (Paulesu et al. 1995, 2005; Paulesu 1997; Romagnoli et al. 2003;

Paulesu, Romagnoli, et al. 2005). Thus, it was assumed that IL1A and IL1B expression was involved in the maintenance of pregnancy in both *C. chalcides* and mammals.

Surprisingly, our transcriptomic analysis reveals no expression of either IL1A or IL1B in both pregnant and nonpregnant *C. ocellatus* uteri and only low (but significant) upregulation of an interleukin 1 receptor (IL1R1). If IL1R1 expression indicates IL1 activity, we cannot locate the source of transcripts, and the lack of IL1A or IL1B expression in the uterus *C. ocellatus* starkly contrasts with that detected in *C. chalcides*. A potential explanation for these conflicting results could be that we were unable to identify IL1A and IL1B transcripts using our sequence identification strategy. That is, IL1A and IL1B may evolve quickly enough that the *Chalcides* transcripts of these genes cannot be reliably aligned to those in our reference genomes. An alternate explanation is that uterine IL1 expression was lost in the evolutionary history of the lineage leading to extant *C. ocellatus*. However, *C. chalcides* and *C. ocellatus* share a common ancestor only ~10.5 Ma (Carranza et al. 2008); that uterine expression of IL1 is also detected in *Zootoca* (*Lacerta*) *vivipara* (Paulesu et al. 2005), a lacertid lizard that shares a common ancestor with scincid lizards (including *Chalcides*) ~175 Ma (Brandley et al. 2008, 2011), and mammals, suggests that uterine IL1 expression is evolutionarily conserved. Although we do not detect IL1 expression, we nonetheless find extensive regulation of other genes that regulate the inflammatory response. The anti-inflammatory cytokine IL11 (Zourbas et al. 2001) is upregulated, whereas the proinflammatory IL8 and IL15 (Zourbas et al. 2001; Challis et al. 2009) are significantly downregulated. Finally, the proinflammatory cytokine high-mobility group box 1 (HMGB1) is significantly downregulated.

Significant upregulation and downregulation of other immune system genes also suggest active regulation of the inflammatory response in the uterus. There is significant downregulation of complement component 3 (C3). C3 plays a key role in activating the immune complement system, a component of the innate immune system that has roles in promoting local inflammation and stimulating a cascade of immune response including elements of the adaptive immune system (Sahu and Lambris 2001; Janssen et al. 2005; Walker et al. 2010). Excessive complement activation may lead to inhibited fetal growth or pregnancy termination in mammals (Caucheteux et al. 2003; Girardi et al. 2006).

However, not all inflammatory immune response genes are regulated to prevent inflammation in the pregnant *C. ocellatus* uterus. For example, phospholipase A2 IB is highly upregulated (PLA2G1B). PLA2G1B is typically expressed in the pancreas to digest dietary phospholipids in the duodenum, but in mammals, it is also expressed in nonpancreatic tissues where it likely plays a role in promoting inflammation by stimulating arachidonic acid release (Nevalainen et al. 2000). Phospholipase A2 expression is also critical for proper implantation in mammals (Dey et al. 2004). On the other hand, function of PLA2G1B may be mediated by the expression of its receptor (Moses et al. 1998) that is not differentially expressed between the pregnant and nonpregnant uteri ($P = 0.91$; data not shown). Regardless, even if PLAS2G1B expression promotes inflammation, its actions are likely regulated by the highly upregulated annexin A4 (ANXA4), a phospholipase A2 inhibitor.

Major histocompatibility loci encode molecules that present antigens to the host's immune system and are a key component in the body's recognition of "self" from "nonself." Downregulating MHC expression in the embryonic tissues is therefore one mechanism by which the embryo can hide from the maternal immune system (Moffett and Loke 2006). However, both MHC Class I and II loci are significantly downregulated in the pregnant *C. ocellatus* uterus (the embryonic tissues were not sampled). Uterine downregulation of MHC Class I expression also occurs in cattle, pigs, and sheep, species with noninvasive epitheliochorial placentae, but in these cases, trophoblast cells merge with those of the uterine epithelia to form binucleate syncytia of embryonic and maternal origin (Davies et al. 2000; Choi et al. 2003; Joyce et al. 2008); downregulation of MHC Class I expression may be a mechanism by which the maternal immune system ignores these allograft cells in mammals. However, there is no evidence of syncytia formation in *C. ocellatus* (but they may form in the very late stages in pregnancy in *C. chalcides* [Blackburn et al. 1998] and some *Mabuya* group skinks [Ramírez-Pinilla et al. 2006; Vieira et al. 2007; Blackburn and Flemming 2011]), and thus, the function of uterine MHC downregulation in *C. ocellatus* remains unclear.

Transport across the Placenta

A majority of the water, organic and inorganic ions, and nutrients used by the embryo in oviparous and lecithotropic viviparous species is provided as yolk within the ovulated egg. By contrast, there is substantial transport of these molecules from the mother to the embryo via the placenta in lizard species with complex placentation ("placentotrophy") (Thompson et al. 2000; Ramírez-Pinilla 2006; Thompson and Speake 2006). This resource allocation strategy must therefore involve substantial changes in the expression of molecular transporter genes, and our transcriptomic analysis of *C. ocellatus* reveals upregulation of many such loci. Placentotrophy has not been directly measured in *C. ocellatus*, and the species ovulates larger eggs (10 mm; Corso et al. 2000) with a larger yolk mass than highly placentrophic species such as *C. chalcides* (3 mm; Giacomini 1891), but light and scanning electron microscopic analysis of placental morphology imply that some placentotrophy occurs in *C. ocellatus* (Corso et al. 2000). Moreover, some placentotrophy has even been documented in primarily lecithotrophic species (Thompson et al. 2000; Thompson and Speake 2006).

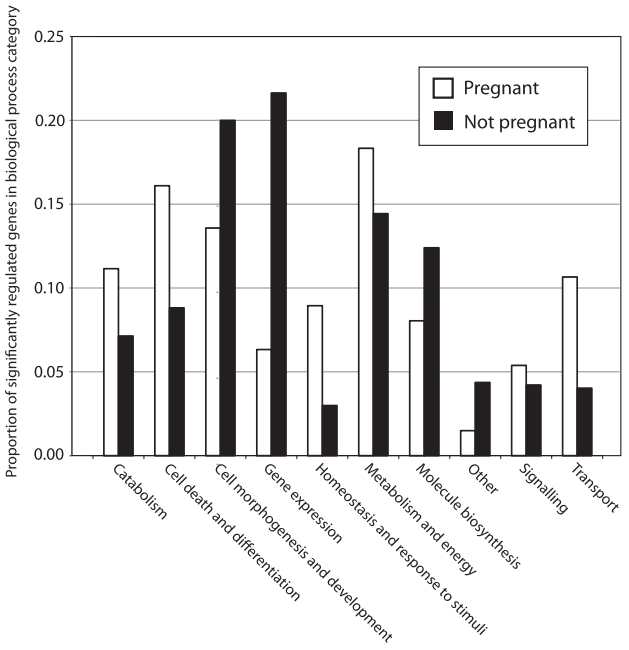

Fig. 1.—Aggregated GO biological processes for significantly regulated genes in the pregnant and nonpregnant uterine transcriptomes. For group descriptions, see text.

Mothers of viviparous species provide much more water to their embryos than oviparous species (Thompson et al. 2000). Water transport is facilitated by aquaporins, cell membrane proteins that selectively regulate water transport. Aquaporins (AQP) are upregulated in the pregnant rat uterus (Lindsay and Murphy 2007) and indeed, AQP1 and AQP3 are also expressed in the uterus of the highly placentotrophic South American scincid lizard, *Mabuya* sp. (Wooding et al. 2010). AQP3, AQP5, and AQP11 are upregulated in the pregnant *C. ocellatus* uterus.

The last third of pregnancy in reptiles is particularly crucial for the provision of nutrients and ions such as calcium (Stewart and Thompson 2000; Herbert et al. 2006) and sodium (Thompson et al. 2001), and most of these ions are transported via transcellular routes (Loffing et al. 2001). The upregulation of 136 genes in 35 families of solute carrier proteins (SLCs; a majority not shown in table 2 but see supplementary information 1, Supplementary Material online) in the pregnant *C. ocellatus* uterus demonstrates a significant shift to increased inorganic and organic molecular transport during pregnancy (see "transport" in fig. 1). These upregulated SLC genes include transporters for inorganic ions (includes Ca^{2+}, Cl^{-}, CO_3^{2-}, H^{+}, HCO_3^{-}, K^{+}, Na^{+}, and PO_4^{3-}), metals (includes Cu^{2+}, $Fe^{2/3+}$, Mg^{2+}, and Zn^{2+}), and organic molecules, including glucose, amino acids, peptides, fatty acids, and carboxylic acids. The three highest upregulated SLC genes are SLC15A1, an oligopeptide transporter, SLC22A17, an organic cation transporter, and SLC2A3, a glucose transporter, which suggest a great transport of organic molecules by the embryo in late pregnancy—a finding supported by physiological studies of other placentotrophic skinks (Thompson et al. 2000; Thompson and Speake 2006).

Lipids represent the primary source of nutrition to the developing embryo, and physiological studies have inferred a net uptake of lipids across the placenta in numerous viviparous species with relatively complex placentae (Thompson et al. 2000; Speake et al. 2004; Thompson and Speake 2006). Lipid transport is likely facilitated by upregulated fatty acid packaging and transport gene families in the uterus of pregnant *C. ocellatus*. The primary functions of fatty acid binding proteins are to facilitate intracellular transport of fatty acids (Chmurzyńska 2006) and are active in the mammalian placenta (Haggarty 2002). Similarly, FABP genes (FABP1—5, 7) are upregulated in pregnant *C. ocellatus*, with FABP1 and FABP7 showing a $\log_2$-fold expression change of 15.1 and 12.8, respectively. Apolipoproteins are transporters of fatty acids, cholesterol, and phospholipids, and five apolipoproteins (APOA1, APOA2, APOA4, APOE, and APOM) are significantly upregulated in the pregnant uterus of *C. ocellatus*.

Uterine Metabolism and Possible Histotrophy Mediated by Lysosomes in the Omphaloplacenta

Uterine Metabolism

As pregnancy progresses, the energy demands to fuel embryonic growth, placental remodeling, and active transport increase sharply, which is reflected in the pregnant *Chalcides* uterus where genes involved with ATP production are highly upregulated. Most of these genes are associated with carbohydrate metabolism, including genes involved in glycolysis (ALDOA, ALDOB, ALDOC, GAPDH, LDHA, PKM2, PGAM1), the citric acid cycle (MDH1, MDH2), electron transport chain (ATP synthases, cytochromes b and c), gluconeogenesis (PCK2), ketogenesis (HMGCS2), and ATP regeneration (CK). One highly upregulated metabolic gene of particular note is glutamate–ammonia ligase (GLUL, also called glutamine synthetase). Glutamine is critical for the synthesis of nucleic acids and sugars as well as cell growth and differentiation (Self et al. 2004), and upregulation of GLUL mirrors the pattern in numerous mammal species where glutamine concentrations in the fetal plasma are far higher than those in the maternal circulation (e.g., Wu et al. 1995; Cetin 2001; Kwon et al. 2003; Manso Filho et al. 2009). Thus, the upregulation of GLUL is probably critical to maintaining both reptilian and mammalian pregnancies. The peroxidases catalase (CAT) and glutathione peroxidases 1, 3–5 (GPX1, GPX3–5) are upregulated in the pregnant *C. ocellatus* uterus. These enzymes function to scavenge reactive oxygen species that are the by-product of oxidative metabolism (Myatt and Cui 2004) and are thus markers of increased uterine metabolic activity during pregnancy.

No study has inferred the precise mechanism by which carbon dioxide produced by the embryo is eliminated by the mother, yet the upregulation of carbonic anhydrase II (CA2) offers an intriguing potential mechanism (Sterling et al. 2001). Carbonic anhydrase II catalyzes the reversible reaction of converting CO_2 and H_2O to HCO_3^- and H^+. In oviparous amniotes, expression of CA2 in the chorioallantois promotes acidification of the eggshell to release calcium carbonate for absorption by the embryo (Stewart and Ecay 2010). Thus, the significant upregulation of this gene in the viviparous *C. ocellatus* uterus suggests that it may play a different role during pregnancy. We hypothesize that CA2 has been co-opted into the role of eliminating CO_2 produced by the embryo in viviparous squamates.

Histotrophy

Viviparous squamate reptiles with complex placentae experience a net uptake of macromolecules during pregnancy (especially lipids; Thompson and Speake 2006). Thus, these species must have the catabolic machinery to digest lipid and protein macromolecules into smaller units that can be actively transported to the embryo via the placenta. Microscopic examination and alkaline phosphatase assays suggest that catabolism and transport of macromolecules occur in the omphaloplacenta (also called the "yolk sac" placenta) of placentrophic lizards (Corso et al. 2000; Adams et al. 2005; Thompson and Speake 2006; Biazik et al. 2009). The omphaloplacenta lies at the abembryonic pole of the embryo/yolk mass and typically consists of well-developed columnar epithelial tissue usually associated with secretory epithelia (Corso et al. 2000; Adams et al. 2005; Thompson and Speake 2006). An extensive array of electron dense structures, interpreted to be lysosomes, occur in the omphaloplacenta of two viviparous *Pseudemoia* skinks (Biazik et al. 2009) and in *C. ocellatus* (Corso et al. 2000, fig. 3*B* in that paper). Although many of the nutrients transported across the placenta to the embryo may derive from the mother's digestive system, the massive upregulation of lysosomal proteases (e.g., cathepsins, LGMN, TPP1) supports the morphological interpretation that histotrophic activity occurs in the omphaloplacenta. We also detected upregulation of lysosomal hydrolases, including hexosaminidases, fucosidases, galactosidases, and sialidase (table 2). Lysosomal hydrolases break down the glycosidic bonds of glycolipids and glycoproteins and frequently process macromolecules digested by proteolytic enzymes (Winchester 2005). The putative presence of lysosomes and the upregulation of lysosomal genes collectively suggest that the omphaloplacenta plays a key role in placentotrophy in *C. ocellatus* but testing this hypothesis will require studies of tissue and cell-specific expression of these genes.

Steroid Hormones

Concentrations of circulating estrogen and progesterone during gestation are extremely varied across both oviparous and viviparous reptile species (Girling and Jones 2003; Murphy and Thompson 2011). In *C. ocellatus*, we found significant downregulation of progesterone (PGR) receptor and estrogen receptor 1 (ESR1) ($\log_2$-fold change = −3.1 and −4.0, respectively). This finding is puzzling because in *C. chalcides*, blood serum progesterone concentrations increase over the course of pregnancy with the highest concentration before birth (Guarino et al. 1998). We did not measure serum levels of progesterone in *C. ocellatus* and thus cannot determine whether the downregulated activity of PGR corresponds to a lower concentration of the hormone during late pregnancy or whether PGR downregulation is a mechanism of functional progesterone withdrawal.

Although serum estrogen has not been measured during pregnancy in any *Chalcides* species, concentrations of circulating estradiol rise during vitellogenesis, peak at the periovulation stage, and then decrease to previtellogenesis concentrations in late pregnancy in the distantly related skink *Niveoscincus metallicus* (Jones and Swain 1996). Thus, low ESR1 expression in the pregnant *C. ocellatus* uterus is not wholly unexpected. The absence or downregulation of PGR and ESR1 in the uterine epithelia is not unprecedented. In mammals, implantation is preceded by a loss of PGR and ESR1 expression in the uterine epithelia (Spencer and Bazer 2002; Spencer et al. 2007; Bazer et al. 2008), and expression in the luminal epithelium and superficial glandular epithelium of PGR and ESR1 is also absent through most of sheep and pig pregnancy, but PR is expressed in endometrial stroma (Geisert et al. 1994; Spencer and Bazer 2002). Mammalian uterine epithelia proliferate in response to progesterone and estrogen, but the actions of these hormones are mediated by paracrine signaling from endometrial stromal cells that do exhibit PGR and ESR1 expression (see Bazer et al. 2008 for review; Cooke et al. 1997). However, this mammalian model of stromal cell–mediated effects of progesterone and estrogen may not be applicable to *C. ocellatus* because our pregnant uterus sample contained stromal tissue, yet PGR and ESR1 expression were downregulated. Sustained PGR and ESR expression also occur in the myometrium of mice and sheep (Spencer and Bazer 2002), but this too appears to differ with *C. ocellatus* given that our tissue sample contained both myometrium and endometrium. In summary, PGR and ESR expression are extremely low in the uterine epithelia of mammals and in the entire uterus of *C. ocellatus*, but the mechanism that explains this phenomenon, whether low progesterone and estrogen levels or some other compensatory mechanism, remains unknown.

Conclusion

This study is the first successful use of RNA-Seq methods to characterize changes in pregnancy-associated gene expression in any squamate reptile and a significant step toward uncovering the genetic mechanisms behind the evolution

of viviparity in reptiles. Our results serve as a baseline for future comparative gene expression analyses. *Chalcides* represents only one of the 100+ origins of viviparity in squamate reptiles, so we are not able assess the generality of our gene expression results. Nonetheless, there are clear parallels between the genetic processes associated with pregnancy in mammals and *Chalcides* in terms of gene expression related to tissue remodeling, angiogenesis, immune system regulation, and nutrient provisioning to the embryo. Although estrogen and progesterone receptors are downregulated in mammalian uterine epithelia, downregulation of these genes in the entire *C. ocellatus* uterus is evidence that the gene expression profiles of pregnant mammalian and reptilian uteri are not identical. Future RNA-Seq analyses of other viviparous and oviparous squamate reptiles will reveal the extent to which our results represent the generality of genetic processes associated with pregnancy in mammals and reptiles and potentially explain why the transition to viviparity is so easy in squamates. Moreover, future studies should localize the expression of highly regulated genes in both maternal and embryonic tissues.

Supplementary Material

Supplementary information 1 is available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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