DETERMINING THE PROTEOMIC PROFILE CHANGES IN *MABUYA*'S SP PLACENTA AND OVARY (SQUAMATA: SCINCIDAE)

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UNIVERSIDAD INDUSTRIAL DE SANTANDER
FACULTAD DE CIENCIAS
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DEDICATORIA

Dedico este libro a mi familia, especialmente a mis abuelitos Juan y Tilcia, y a mis tías Carolina y Milena, por su paciencia y compañía durante mi formación personal y académica.

A mi mami Olga, por ser siempre una fuente de consejos excelentes en mi vida, por ser mi fuerza para lograrlo, mi palabra de ánimo cada día, aunque la distancia nos separe.

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RESUMEN

TÍTULO: DETERMINACIÓN DE CAMBIOS EN EL PERFIL PROTEOMICO EN PLACENTA Y OVARIO DE *MABUYA* SP (SQUAMATA: SCINCIDAE) *

AUTOR: YURANY NATHALY HERNÁNDEZ DÍAZ**

PALABRAS CLAVE: VIVIPARIDAD, GESTACIÓN, PROTEOMICA, PLACENTOTROFÍA, DESARROLLO EMBRIONARIO

Los reptiles son el grupo más diverso entre los vertebrados, proporcionan un sistema integral para estudios comparativos sobre metabolismo, fisiología animal y biología del desarrollo. Sin embargo, los datos moleculares disponibles son limitados y sólo recientemente han comenzado a llamar la atención en las ciencias "ómicas". *Mabuya* sp es una especie vivípara placentotrofica colombiana con importantes características reproductivas, incluido el desarrollo de huevos microlecitos, luteólisis temprana y el desarrollo de una placenta altamente especializada implicada en el intercambio respiratorio y la transferencia de todos los nutrientes (iones, lípidos y nitrógeno, como un índice de proteínas) necesarios para el desarrollo del embrión. Nuestro objetivo fue identificar las proteínas que se expresan diferencialmente en el ovario y la placenta de *Mabuya* sp durante la gestación temprana, media y tardía, sus posibles rutas metabólicas y procesos biológicos. Se realizó un análisis proteómico comparativo durante la gestación en ambos tejidos por SDS-PAGE, 2D-PAGE y MALDI-TOF. Diferencial (t-Student $P < 0,05$). La identificación de proteínas expresadas en la placenta y el ovario están relacionadas con procesos como la estructura y movimiento celular y energía entre otros. Las proteínas que se encontraron en el ovario se asocian principalmente con el desarrollo folicular y su regulación. En la placenta, en particular durante mediados y finales de la gestación cuando está es madura y altamente especializada, la expresión de proteínas está involucrada con funciones como metabolismo de los nutrientes, el transporte, la síntesis de proteínas y el desarrollo embrionario. Este trabajo proporciona información detallada preliminares sobre las proteínas expresadas y sus mecanismos fisiológicos en la placenta de *Mabuya* sp y el ovario durante la gestación.

* Trabajo de grado.

** Facultad de Ciencias. Escuela de Biología. Director: Martha Patricia Ramírez Pinilla, *Ph.D*. Codirector: Rodrigo Torres Sáez, *Ph.D.*

ABSTRACT

TITLE: DETERMINING THE PROTEOMIC PROFILE CHANGES IN *MABUYA*'S SP PLACENTA AND OVARY (SQUAMATA: SCINCIDAE)

AUTHOR: YURANY NATHALY HERNÁNDEZ DÍAZ**

KEYWORDS: VIVIPARITY, GESTATION, PROTEOMICS, PLACENTOTROPHY, EMBRYONIC DEVELOPMENT.

Reptiles are the most diverse group among vertebrates, providing an integrated system for comparative studies on metabolic, animal physiology and developmental biology. However, the molecular data available is limited and only recently have started to call attention in the "omics" sciences. *Mabuya* sp is a viviparous placentrotrophic colombian skink with important reproductive features, including microlecithal eggs, early luteolysis, and the development of a highly specialized placenta implicated in respiratory exchange and the transference of all nutrients (ions, lipids and nitrogen, as an index of protein) necessary for embryo development. Our aim was to identify proteins that are differentially expressed in the ovary and placenta of *Mabuya* sp during early, mid and late gestation, their possible metabolic pathways and biological processes. We carried out a comparative proteomic analysis during gestation in both tissues by SDS-PAGE, 2D-PAGE and MALDI-TOF. Differential (Student's t-test $P<0.05$) ovarian and placental protein identification were related to housekeeping processes as cell structure, movement and energy. Proteins found in ovary are mainly associated with follicular development and its regulation. In the placenta, particularly during mid and late gestation when it is mature and highly specialized, protein expression is involved with functions like nutrient metabolism, transport, protein synthesis and embryonic development. This work provides preliminary insights about the proteins expressed and their physiological mechanisms in *Mabuya*'s sp placenta and ovary during gestation.

* Bacherlor Thesis

** Science Faculty Department of Biology. Director: Martha Patricia Ramírez Pinilla, *Ph.D*. Codirector; Rodrigo Torres Sáez, *Ph.D.*

1. INTRODUCTION

The evolution of placentation in vertebrates is linked to the evolution of viviparity, therefore parity mode and embryonic nutrition are individual but not independent traits of reproductive patterns in vertebrates. Among most amniotes, parity mode is a conserved trait classified into oviparous species (egg laying as for example in birds, crocodilians, turtles, and monotremes) and live bearing species (e.g. in metatherian and eutherian mammals). On the other hand, pattern of embryonic nutrition is classified into lecitrotrophy (embryo/neonate nutrition derived from reserves accumulated in the yolk) and matrotrophy (nutrition from the mother) depending on the source of nutrients for the embryo (Daniel G. Blackburn, 2000).

Viviparity has evolved ~ in 115 Squamata lineages (lizards and snakes) independently (Sites, Reeder, & Wiens, 2011); for this reason, they are particularly important due to the exhibition of a complex mosaic of different parity modes and embryonic nutrition patterns even in the same family, and even between species populations (Daniel G. Blackburn, 2006; Sites *et al*., 2011). Consequently, Squamata provides an integrated system for comparative studies on metabolic, animal physiology and developmental biology. However, the molecular data available for reptiles is limited and only recently have begun to draw attention in the "omics" sciences (Tollis, Hutchins, & Kusumi, 2014).

Few studies from the “omics” sciences has been carried out in squamate viviparous species and have significantly advanced our fundamental knowledge about aspects involved in viviparity and its physiology, including processes of angiogenesis (Murphy, Belov, & Thompson, 2010), hormonal signaling (Griffith *et al*. 2016a), gestation (Brandley, Young, Warren, Thompson, & Wagner, 2012; Paulesu *et al*., 2001) and genomic imprinting (Griffith *et al*. 2016b).

Specialization and placentation related to viviparity are unique in Scincidae family among reptiles and might be a result of convergent evolution with mammals (Brandley *et al*., 2012; Van Dyke, Brandley, & Thompson, 2014). Within this family, viviparous skinks of the genus *Mabuya* exhibit extreme placentotrophy and the greatest complexity in morphology and physiology of their allantoplacenta (derived from chorionic and allantoic extraembryonic membranes) among squamates (Daniel G. Blackburn & Flemming, 2009; Daniel G. Blackburn & Vitt, 2002; Jerez & Ramírez-Pinilla, 2003). Related to these features, *Mabuya* have a highly conservative morphology (Miralles & Carranza, 2010), early sexual maturity, long gestation period (9 to 10 months), and microlecithal eggs (1-2 mm diameter) (D G Blackburn, Vitt, & Beuchat, 1984; Daniel G Blackburn & Vitt, 1992; Jerez & Ramírez-Pinilla, 2001).

Mabuya ovarian features have been associated to the high degree of placental complexity of these species (Gómez & Ramírez-Pinilla, 2004, Vieira *et al*., 2010). Among them, the corpus luteum (involved in gestation maintenance and development due to high progesterone production) begins luteolysis in early gestation, during gastrula-neurula embryonic stages (Gómez & Ramírez-Pinilla, 2004). Therefore, maintenance of gestation from pharyngula stages to the end, seems to be related to secretion of extra-luteal progesterone, possibly from placental origin as the allantoplacenta starts to develop at this stage (Jerez & Ramírez-Pinilla 2003).

Since *Mabuya* produces eggs with a minimal quantity of yolk, intraoviductal embryo development depends on the transference of nutrients through the specialized placenta (Ramirez-Pinilla, 2006). Wooding *et al*. (2010) found by immunocytochemistry the localization of multiple transport routes in the allantoplacenta of *Mabuya* sp. They found the expression of actin, lectin, calcium binding proteins [CBP], glucose transporters, and water channel proteins that demonstrated restricted distributions in specialized areas of the allantoplacenta

(Leal & Ramírez-Pinilla, 2008). This allantoplacenta is also implicated in the transfer of ions as calcium, potassium, sodium, magnesium, iron; lipids as cholesterol, vitamin E, fatty acids, triacylglycerol, phospholipids, free fatty acids and nitrogen as total protein content (Ramírez-Pinilla, Rueda, & Stashenko, 2011).

Proteomics provides an ideal tool to study the complete set of proteins expressed in a biological compartment (cell, tissue, organ) at a particular time, under a particular set of conditions (Beranova-Giorgianni, 2003), besides proteomics leads to protein identification and quantification, their interactions, modifications and locations. The most widespread strategy for studying global protein expression employs two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (Rotilio *et al*., 2012) coupled to mass spectrometry, this combination shows feasibility and efficiency (Susnea *et al*., 2012).

There are no studies about the ovary and placenta protein expression in *Mabuya* sp during gestation, particularly once the corpus luteum is degenerated. In this study, our aim was to identify proteins that are being differentially expressed in the ovary and placenta of *Mabuya* sp during different stages of gestation in order to provide information about their biological and metabolic pathways. This protein identification is based on the hypothesis that most of the proteins expressed in *Mabuya* sp evaluated organs are similar to those known in other amniotes (Keverne, 2014; Renfree, Suzuki, & Kaneko-Ishino, 2012; Saben *et al*., 2014).

2. MATERIAL AND METHODS

2.1 STUDIED POPULATION

Mabuya is a neotropical viviparous skink genus widely distributed throughout Caribbean islands, Central and South America. Currently, most of the Colombian continental populations of the genus *Mabuya* lack a clear taxonomic identity. Pinto-Sánchez *et al*. (2015) identified at least four new Colombian species candidates (candidate species I to IV). We will focus our work on population of candidate sp. IV which has been previously studied (Jerez & Ramírez-Pinilla, 2001, 2003; Gómez & Ramírez-Pinilla, 2004; Ramírez-Pinilla, 2006; Ramírez-Pinilla *et al*., 2006, 2011; Leal & Ramírez-Pinilla, 2008; Vieira *et al*. 2007, 2010; Wooding *et al*. 2010).

2.2 ANIMAL TISSUES

Lizards were handle captured in the municipality of Curití, department of Santander, Colombia (altitude 1409 m). Snout-vent length (SVL) was registered. Females were killed by intrathoracic injection of lidocaine 2% and dissected. Ovaries and oviducts with the extraembryonic membranes of each embryonic chamber were extracted and kept individually in cryogenic storage vials at -70 °C. Females bodies and embryos were fixed in formalin 10%, preserved in a graded ethanol series (ethanol 30%, 50%, 70%) and deposited in the Herpetological Collection of de Museo de Historia Natural of the Universidad Industrial de Santander. Embryos were classified in development stages under stereo microscope according to the Dufaure & Hubert (1961) developmental stage table.

Extraembryonic tissues in gastrula stage (early gestation, n=7), extraembryonic tissues during mid-gestation (n=10) and extraembryonic tissues during late gestation

(n=8) were obtained from eight pregnant females (n=8) between 2014-2015. Ovarian tissues were collected from females in early gestation (n=3), mid-gestation (n=3) and late gestation (n=2).

2.3 PROTEIN EXTRACTION

Frozen tissues were washed in cold isotonic phosphate-buffered saline solution. The cell lysis, precipitation, protein amount and spot resolution parameters were evaluated in order to design an adequate protocol for optimizing the sample preparation. Each sample was sonicated using an ultrasonic processor (Cole-Parmer U.S.A®) in lysis buffer (Urea 7 M, Thiourea 2 M, Dithiothreitol (DTT) 100 mM, Tris 40 mM, PMSF 1 mM, EDTA 1 mM, 4% Chaps, 1% Ampholyte, Sodium orthovanadate 1 mM). Placental proteins were precipitated with dichloromethane/methanol according to Centlow *et al*. (2010) with some modifications, samples were centrifuged at 14.000g for 10 minutes at 4 °C. Three volumes of methanol were added and centrifuged again at 18.000g for 10 minutes at 4 °C (twice). Ovarian proteins were precipitated with acetone and centrifuged. The protein concentration was determined by Bradford (1976) method using bovine serum albumin as standard.

2.4 SDS-PAGE

Each type of sample (placental and ovarian) was mixed with loading buffer (25% Tris-HCl 0.5 M, 20% Glycerol, 10% SDS, 10% 2-Mercaptoethanol, 5% Bromophenol blue). 20 µL were loaded on 12% SDS-PAGE (Laemmli, 1970). The gel was stained with coomassie brilliant blue R-250. The molecular weight of the samples was determined by comparing electrophoretic mobility with PageRuler^{TM} unstained protein ladder (Thermo Scientific®).

2.5 2D-PAGE

Proteins were analyzed by 2-DE as described by O'Farrel (1975) and modified by Görg *et al*. (2004). A total of 180 µg of placental and 90 µg of ovarian proteins were mixed with rehydration buffer (Urea 8 M, 2% Chaps, DTT 50 mM, 0.2% Ampholyte and 0.001% Bromophenol blue) and loaded onto 7 cm pH ranges 3-10 non-linear IPG gel strip (BioRad®). Isoelectric focusing (IEF) was conducted with a Protean IEF cell. After IEF, the IPG-strips were equilibrated for 15 minutes in equilibration buffer (Urea 6 M, Tris-HCl 0.375 M pH 8.8, 2% SDS, 20% Glycerol), 2% DTT was added in the first step to reduce disulfide bonds and 2% iodoacetamide to the second equilibration step to block sulfhydryl groups.

Second dimension was carried out on 12% SDS-PAGE gels at 150 V constant power until the bromophenol blue reached the bottom of the gel. 2-D SDS-PAGE Standards (Bio-Rad®) was used for spot matching and molecular weight/ isoelectric point determination. For each embryonic stage (early, mid and late), a total of three independent placental samples (n=3) were used for running three times 2-DE gels to reduce the experimental noise. Because the number of available ovarian samples, just one ovarian tissue sample from early, mid and late gestation period were run in 2-DE gels.

2.6 PROTEIN DETECTION

Placental proteins were fixed in 50% v/v methanol and 2% v/v phosphoric acid overnight, and then transferred to a solution with 33% v/v methanol, 17% w/v ammonium sulphate and 3% v/v phosphoric acid. Gels were stained with coomassie brilliant blue G-250. Ovarian proteins were fixed in 10% v/v methanol and 7% v/v phosphoric acid and stained with Sypro Ruby (Bio-Rad®). Staining gels images were acquired using a Gel Doc XR (Bio-Rad®). The digitized images were analyzed with PDQuest Advanced Software V.8.0.1 (Bio-Rad®) for spot detection, spot matching and spot quantification. Differences on the spots mean intensity were evaluated by

student's t-test between developmental stages in placenta, P-values below 0.05 were considered as significant differences. Quantitative analyses (1.5-fold change, indicates increased or decreased protein expression over 1.5 times the expression level in one group) were evaluated between ovarian samples.

2.7 TRYPTIC DIGESTS OF GEL-SEPARATED PROTEINS

Significantly differentially expressed protein spots were subjected to trypsin digestion and MALDI-TOF mass spectrometry. Protein spots were manually excised and destained with 50% acetonitrile and 50 mM ammonium bicarbonate. Each dried piece was dehydrated with 50 mM ammonium bicarbonate containing 13 ng/µL trypsin (Sequencing Grade Modified, Promega®) at 4 °C during 30 minutes and incubated at 37 °C for 17 hours. Tryptic peptides were mixed with α-cyano-4-hydroxicinnamic acid (HCCA) as matrix and placed onto a ground steel target. The identification of proteins was determined using a time-of-flight mass spectrometer (Bruker).

The peptide masses were compared with the theoretical peptide masses of all available proteins from all related species on Mascot database engine freely available on the website of Matrix Science (http://www.matrixscience.com/), criteria used to accept identification included the extent of sequence coverage, the number of peptides matched, the probability score, and protein scores greater than 65 were considered significant ($P<0.05$) (Perkins, Pappin, Creasy, & Cottrell, 1999).

2.8 BIOINFORMATICS ANALYSIS

Homologous of the differentially expressed proteins in a viviparous well characterized mammalian species (Homo sapiens) were mapped, since there is no sequenced a complete genome for a viviparous species of Squamata. Accession lists generated by results from the Mascot searches were imported into the STRING 10.0 database (Szklarczyk *et al*., 2015). The sample data were used to build

predictive proteomic pathways and study the predicted functional partners of known protein–protein interactions (http://string-db. org/).

3. RESULTS

In this work, we performed a comparative proteomic study in reproductive tissues (ovary and placenta) of *Mabuya* sp during three stages of gestation. The stages of gestation were determined by the developmental stages of the embryos in the pregnant females. They correspond to: early gestation with embryos at gastrula stages (Figure 3A, B); mid gestation with embryos at stage 34 (limb bud stages, characterized by organogenesis in progress, closed gill slits, circular eyelid and lower jaw develop, Figure 4A, B); and late gestation with fetuses at stage 39 (preparturition stages, characterized by the presence of external nares and tympanum, fingers and claws are well developed and independent body pigmented scales, Figure 5A, B).

It is worth to highlight that sample preparation is essential in proteomic analyses, which should be as simple as possible to increase reproducibility, and to minimize protein modifications, because it might result in artefactual spots on 2-D gels (Görg *et al*. 2000). For this reason, an adequate protocol is required in order to optimize the tissue preparation, e.g. the placenta is a difficult tissue to work with for proteomic studies, because it contains large amounts of lipids and glycogen, which are well known to interfere with the isoelectric focusing in 2-DE (Rabilloud & Lelong 2011).

In order to obtain an optimal protein separation on 2-DE, the design of a standard protocol for sample preparation in both tissues aimed to minimize experiment-to-experiment variations was developed (with the available instruments). For this protocol, four critical parameters were considered: tissue homogenization/extraction

(potter-Elvehjem PTFE pestle and ultrasonic processor), solvent precipitation for removing interferences (the following organic solvents were used: acetone, acidified acetone and dichloromethane/methanol), protein amount (quantified by Bradford method) and spot resolution. The glycogen removal from the samples by centrifugation was crucial for the final proteome maps. Homogenization with ultrasonic homogeinezer in combination with dichloromethane/methanol proved to be the optimal procedure to obtain less contamination (Centlow *et al*. 2010) and more protein quantity (Anova P>0.05).

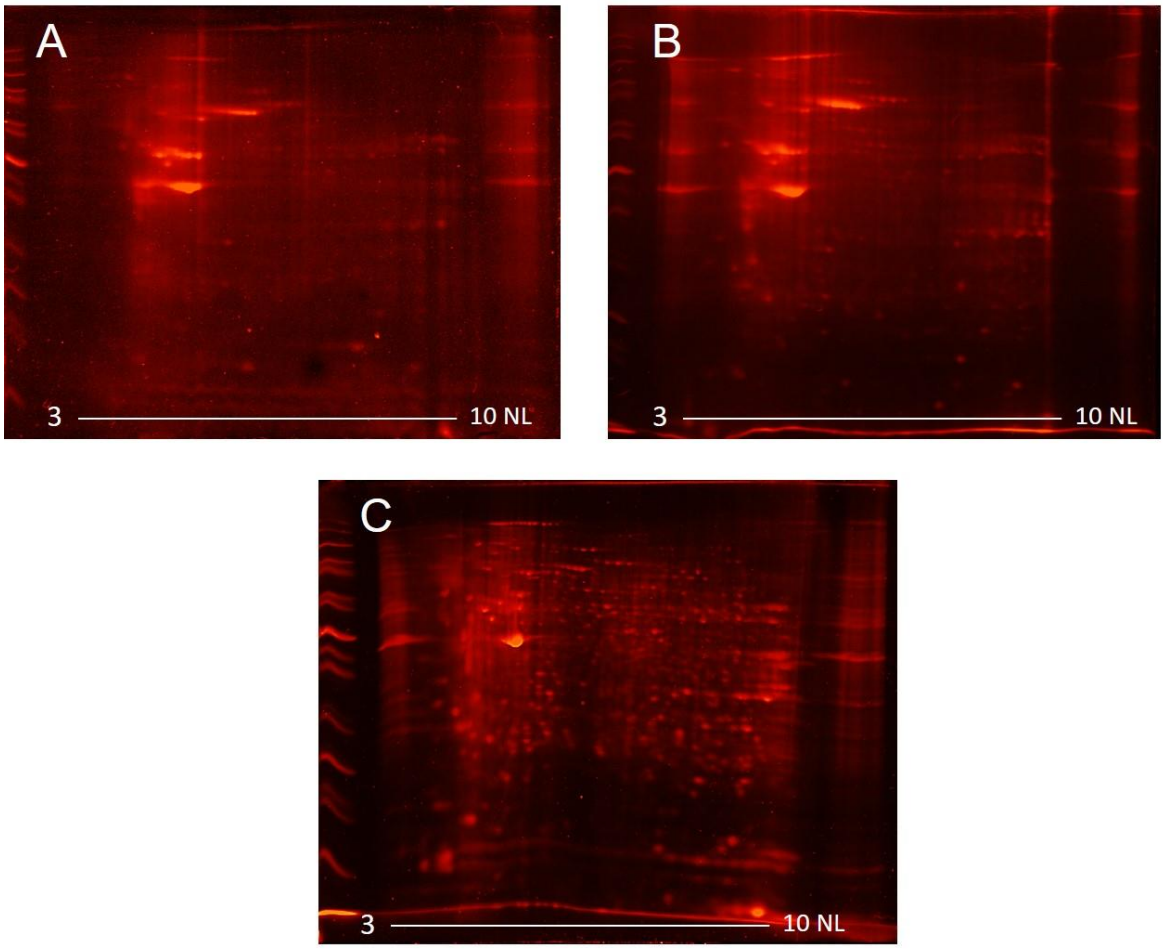

Figure 1. Representative 2-DE gels (12%, pH 3-10 NL) images of ovary protein extracts in embryonic development of *Mabuya* sp stained with sypro-ruby. A. Proteins expressed at gastrula stage (early gestation). B. Proteins expressed at 34 stage (mid gestation) C. Proteins expressed at 39 stage (late gestation).

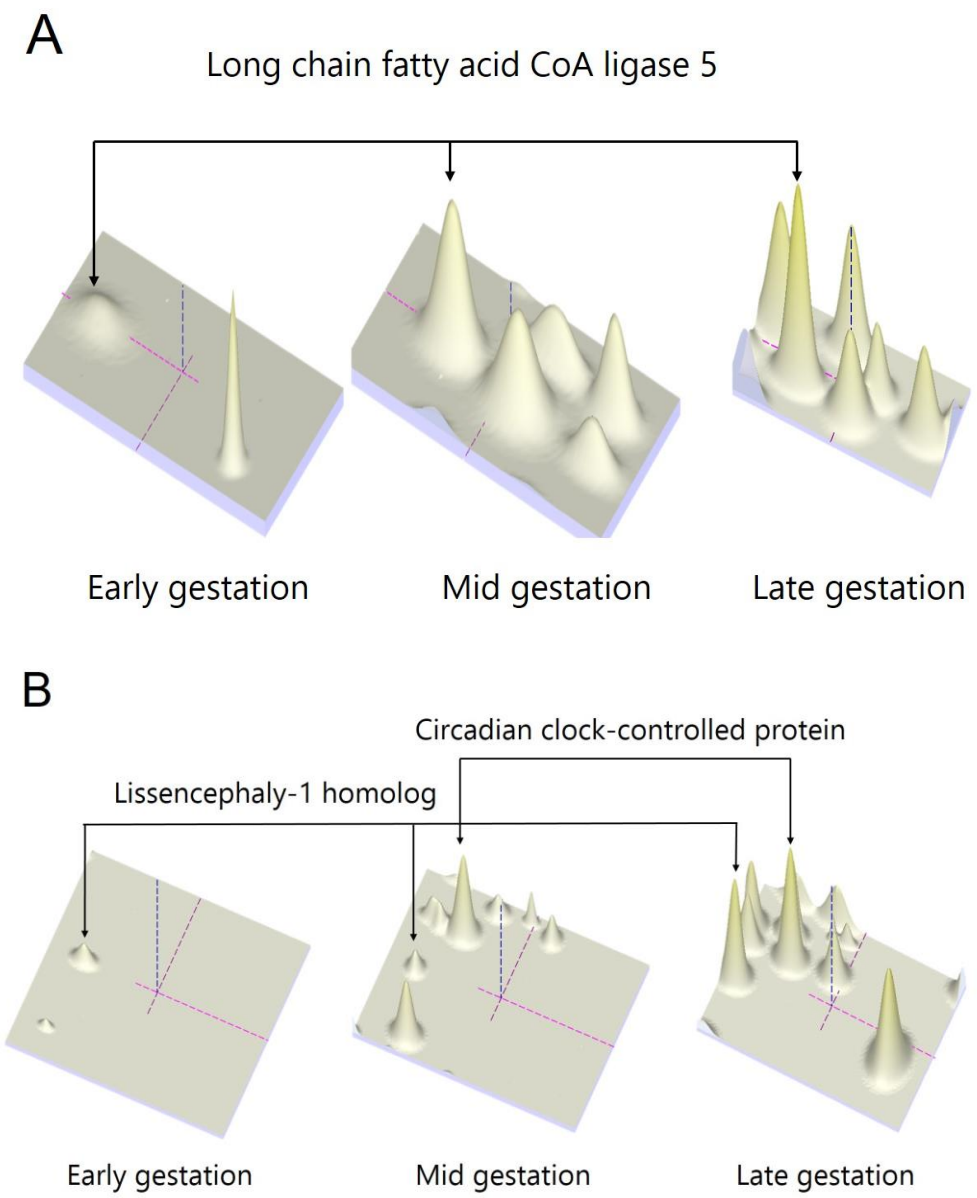

Figure 2. Representative 3D spot view of proteins up and downregulated in *Mabuya* sp ovarian tissues with PdQuest Advanced v8.0 software. A. Upregulation in mid and late gestation of long chain fatty acid CoA ligase 5 compared to early gestation. B. Upregulation of lissencephaly-1 homolog and circadian clock-controlled protein in mid and late gestation compared to early gestation.

Table 1. Up and downregulated proteins identified by MALDI-TOF analysis in ovary of *Mabuya* sp embryonic stages.

SSP	Access number[a]	Protein name	Score[b]	MW	PI	Cov.%[c]	Expression level[d]	Biological process
				Cell morphology and motility				
3504	Q9UVW9	Actin, gamma	82	41809	5.45	25	- + +	Structure, cell motility, ATP-binding
2504	Q964E3	Actin, cytoplasmic	68	42226	5.3	26	+ + -	Structure, cell motility, ATP-binding
2505	P02574	Actin, larval muscle	93	42159	5.3	39	- - +	Muscle contraction ,cytoskeleton structure, cell motility
2502	Q3ZCJ7	Tubulin alpha-1C chain	72	50510	4.96	23	- + +	Microtubule-based process, GTP-binding, nucleotide binding
				Energy metabolism				
2602	Q05825	ATP synthase subunit beta, mitochondrial	88	54074	5.14	27	+ - +	ATP synthesis, Hydrogen ion transport, Ion transport, Transport
8001	Q6DKE1	Cytochrome c	79	11802	9.54	43	- - +	Electron transport, Respiratory chain, Transport
4608	Q8JZR0	Long-chain-fatty-acid--CoA ligase 5	67	77013	6.74	21	- + +	Fatty acid metabolism, Lipid metabolism
				Amino-acid biosynthesis				
1203	Q6BXC7	Enolase-phosphatase E1	75	27271	4.75	28	- + +	Amino-acid biosynthesis, methionine biosynthesis

				Biological rhythm				
4305	O76879	Circadian clock-controlled protein	67	29388	6.9	18	- + +	Biological rhythms
				Follicular development and regulation				
4401	Q9PTR5	Lissencephaly-1 homolog 1	73	46557	6.65	11	- + +	Cell division, Mitosis, Transport, Ce
				Transcription regulation				
8601	Q6ZN06	Zinc finger protein 813	69	73841	9.5	15	+ - +	Transcription, Transcription regulation
8205	Q9N1Q5	Nuclear autoantigen Sp-100 (Fragment)	71	28441	9.68	41	- + +	Transcription, Transcription regulation

[a] UniProt serial number.

[b] Protein score higher than 65 were considered statistically significant ($P<0.05$).

[c] Sequence percent coverage.

[d] Indicates protein differential expression between gastrula (early gestation), 34 (mid gestation) and 39 (late gestation) developmental stages respectively. Protein expression: (+) protein upregulated, (-) protein downregulated.

About 180 µg and 90 µg for placental and ovarian tissues respectively was the appropriate amount of protein to be loaded per strip (taking into account spots quality). In other studies, this protocol has shown to be reproducible in the number of detected spots. But having into account that the maximum amount of protein obtained from placenta was around 200 µg and 100 µg for ovarian tissues it was no possible to run technical replicates. Therefore, in this case, experimental design (three individual placental tissues to run 2-DE gels and one 2-DE gel from each ovarian tissues per gestation groups) were correlated with biological variation to do preliminary but statistically significant conclusions. A narrow degree of biological variation (correlation coefficient) was observed from experiment to experiment in the process of 2-DE (Table 3). The broad technical variation from the coefficient of variation (Table 3) was probably related to the changes through pregnancy.

On the other hand, with the extracted protein in samples that were not enough to run 2-DE gels, 1D SDS-PAGE experiments were carried out from three independent samples per gestation group with its correspondingly three replicates (since the amount of protein was sufficient), considering taking into account both technical and biological variation.

Proteins involved in pregnancy of *Mabuya* sp in the different gestation stages were separated, analyzed and compared for the first time. SDS-PAGE and 2-DE gel images showed favorable repeatability for each stage. Each spot clearly matching all three placental 2-DE gels from each gestation group was selected to build the master gel (a synthetic image containing all the information about the spots in the MatchSet). For this reason, a total of 318, 45 and 34 spots were detected in the placental master gel image, e.g. 350, 347 and 339 spots were detected on each 2-DE gel from placental tissues in early gestation respectively. A total of 57, 128 and 419 spots were detected in each ovarian gel on early, mid and late gestation, correspondingly (Figure 3,4 and 5).

Comparison of spots in 2-DE gels between groups for each tissue revealed proteins with differential expression (up or down regulated between stages, Figure 2 and 6) and proteins expressed just in one stage. We succesfully identified by MALDI-TOF 14 proteins differentially expressed in the ovary and 37 proteins expressed differentially in the placental tissues (Table 1, 2).

Proteins extracted from ovary were classified according to their biological function (Table 1): (1) cell morphology and motility (2) energy metabolism, (3) amino-acid biosynthesis (4) biological rhythms, (5) follicular development and regulation, (6) transcription regulation.

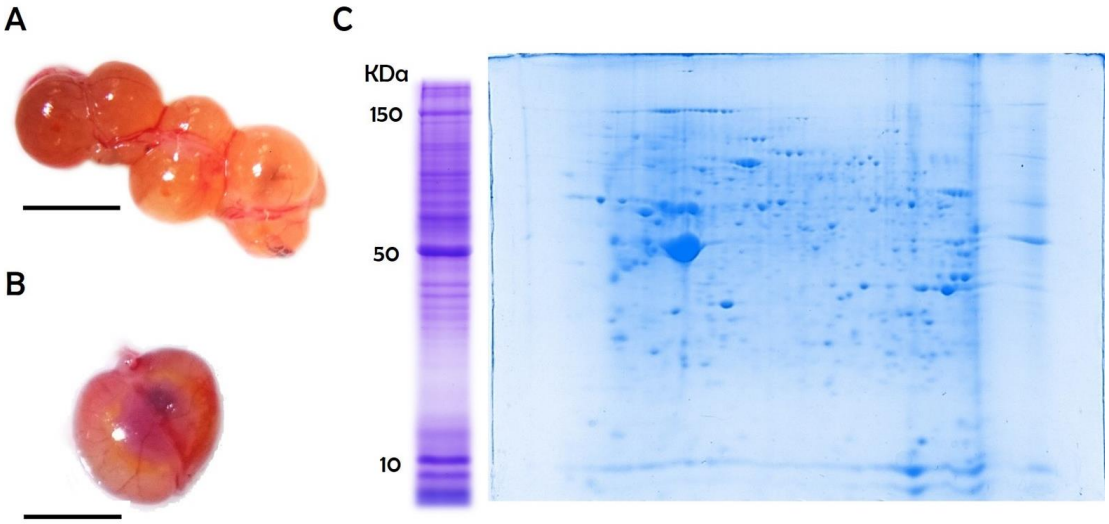

Figure 3. Extraembryonic tissues and embryo at gastrula stage and representative gels images of placental protein extracts in embryonic development of *Mabuya* sp. A. Extraembryonic tissues, embryo at gastrula stage. B. Embryonic chamber in early gestation. C. Proteins expressed at gastrula developmental stage in 2-DE gels (12%, pH 3-10NL stained with coomassie brilliant blue G-250) with monodimensional profiles stained with coomassie brilliant blue R-250. Scale bar 2 mm.

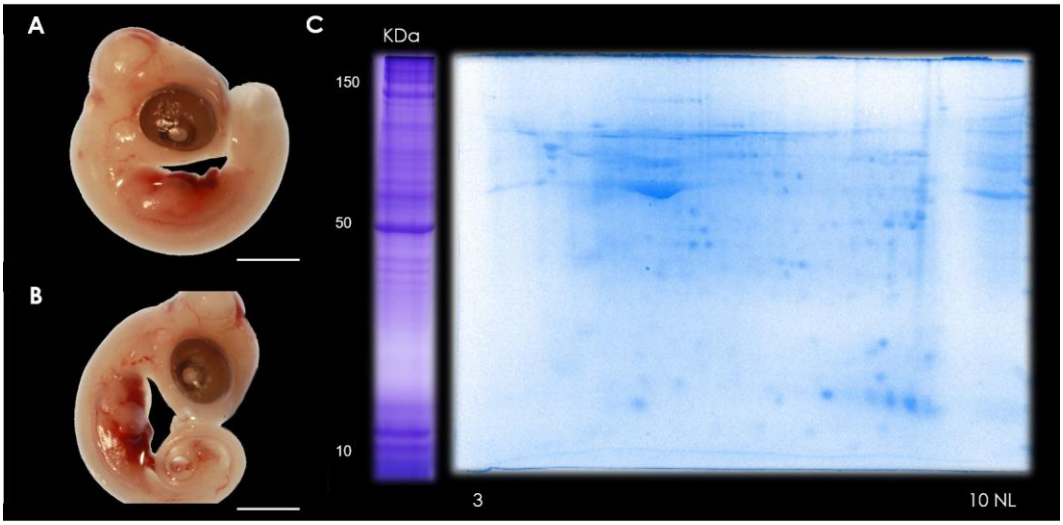

Figure 4. Embryo at mid gestation and representative gels images of placental protein extracts in embryonic development of *Mabuya* sp. A, B. Embryo at 34 developmental stage. C. Proteins expressed in mid gestation in 2-DE gels (12%, pH 3-10NL stained with coomassie brilliant blue G-250) with monodimensional profiles, stained with coomassie brilliant blue R-250. Scale bar 5 mm.

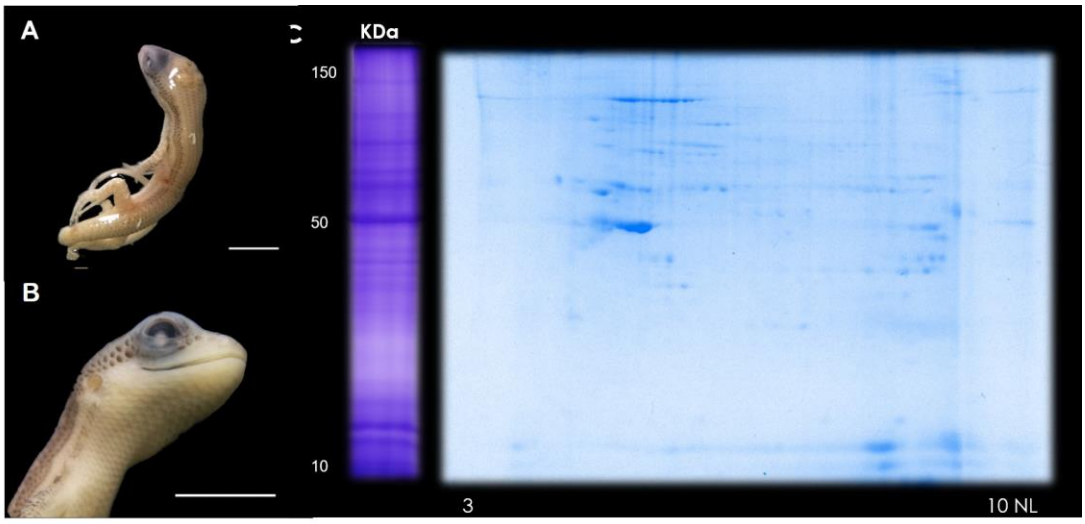

Figure 5. Embryo at preparturition gestation and representative gels images of placental protein extracts in embryonic development of *Mabuya* sp. A. Embryo at 39 developmental stage, scale bar 5mm B. Embryo at 39 developmental stage with emphasis on the pigmented scales in the head, scale bar 5 mm. C. proteins expressed in late gestation in 2-DE gels (12%, pH 3-10NL stained with coomassie brilliant blue G-250) with monodimensional profiles stained with coomassie brilliant blue R-250.

Cell morphology and motility proteins found in ovary (actin and tubulin, Table 1) in mid-late gestation seem to be related to follicular growth. The high expression of circadian clock-controlled protein, lissencephaly-1 (Figure 2B) and nuclear autoantigen SP100 (Table 1) suggest that processes as cell growth and differentiation, e.g. the regression of the corpus luteum and previtellogenesis in mid gestation, and vitellogenesis at the end of gestation, are being regulated and controlled to prepare a new ovulation once gestation had finished. The energy to supply this processes is given by lipids metabolism, due to the high amounts of fatty acids activation by the long chain fatty acid CoA ligase 5 (Table 1) as well upregulated in mid and late gestation (Figure 2A).

Analysis at early gestation in placental tissues showed the highest protein expression because at this stage is not possible to separate the embryo from the placental membranes; so, it includes proteins from the uterus and the embryo (Figure 3A, B). According to their biological function, proteins extracted from placental tissues were classified into the following groups (Table 2): (1) cell morphology and motility (2) energy metabolism, (3) nutrient metabolism and transport, (4) cellular homeostasis, (5) protein synthesis, processing and regulation, (5) protein transport, (6) signaling pathways, (7) embryonic development, (7) proteins foldings, (8) protein modifications and (8) others processes.

Proteins related to cell morphology and motility as actin, tubulin, adseverin, desmin, alpha-actinin-3 were highly expressed during early gestation (Table 2). These proteins are associated to cytoskeletal reorganization, mostly when the single-layered blastula is restructured into three germ layer structure (ectoderm, mesoderm and endoderm). As well, gastrulation is also regulated by expression of zinc finger protein 17 (Figure 6A), a transcription factor essential for ectoderm development during early gestation (Table 2).

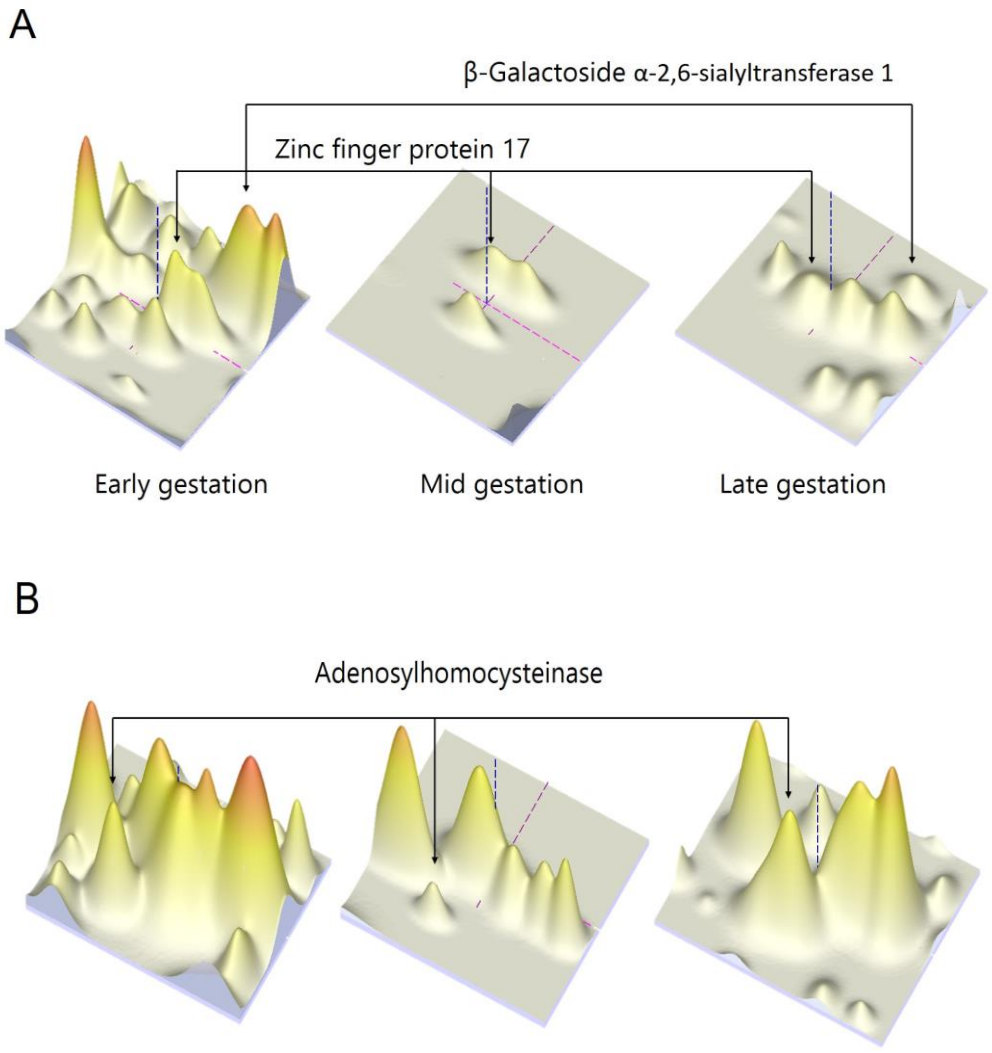

Figure 6. Representative 3D spot view of proteins up and downregulated in *Mabuya* sp placental tissues with PdQuest Advanced v8.0. A. Upregulation in early gestation of zinc finger protein 17 and β-galactoside α-2,6-syaliltransferase 1 compared to mid and late gestation. B. Downregulation of adenosylhomocysteinase in mid gestation compared early and late gestation.

When mid gestaton has advanced and the allantoplacenta is actively transferring nutrients to the embryo, upregulation of proteins related to energy metabolism are detected. Sterol O-acyltransferase 1, a protein implicated in lipid metabolism (cholesterol, steroids and sterol) when the glycolytic proteins alpha-enolase and fructose-biphosphate aldolase were downregulated during mid and late gestation

(Table 2), suggest that lipid metabolism is the main energy pathway during gestation in *Mabuya* sp placental tissues.

Similarly, the placenta is related to the metabolism of nutrients and its transport. Expression of adenosylhomocysteinase (Figure 6B), tyrosine decarboxylase 3 and hemoglobin subunit alpha (Table 2), proteins necessary in cellular amino acid biosynthesis and gas exchange between the maternal uterus and the extraembryonic membranes were higher when the *Mabuya*'s sp allantoplacenta is already specialized from mid gestation. On the other hand, progestin receptor alpha-B (which binds to progesterone, Pg) was found upregulated in placental tissues during early and mid gestation (Table 2), possibly, the high expression of Pg until mid gestation can help the placenta to maintain the progress of gestation, decreasing myometrial contractility. Later, at the end of gestation progestin receptor alpha-B was downregulated apparently by the close up of the labour.

Proteins involved in ribogenesis (40S ribosomal protein S19-1 and 39S ribosomal protein L15, mitochondrial) are highly expressed in placenta at early pregnancy in *Mabuya* sp (Table 2). Protein synthesis, regulation, transport and its signaling is vital to development of any cell. Heat shock proteins (HSPs) including, heat shock 78 kDa glucose-related protein, protein bobber 2 and heat shock congnate 71 KDa protein are chaperones also upregulated in early gestation (Table 2). Varied HSPs expression seemed to be very essential in critical steps of early embryonic development, regulating processes as cell movements and proliferation during gastrulation. When the development has progressed and the placenta is highly specialized at the end of gestation, the upregulation of heat shock congnate 71 KDa protein (Table 2) might be related to the checking and the correct assembly of proteins to maintain their accurate function, and perhaps to prevent oxidative stress that brings pregnancy.

Table 2 Up and downregulated proteins identified by MALDI-TOF analysis in placenta of *Mabuya* sp embryonic stages.

SSP	Access number[a]	Protein name	Score[b]	MW	PI	Cov.%[c]	Expression level[d]	Biological process
Cell morphology and motility								
3403	P10365	Actin	80	41837	5.44	21	+ - +	Structure, cell motility, ATP-binding
3402	Q964E0	Actin, cytoplasmic 1	91	42208	5.3	47	+ + -	Cell juction assembly, movement, chromatin remodeling
3401	Q90X97	Actin, alpha skeletal muscle	147	42376	5.23	43	+ + -	Skeletal muscle fiber development, mesenchyme migration, muscle contraction
3401	P10995	Actin, alpha skeletal muscle 2	126	42304	5.22	45	+ + -	Structure, cell motility, ATP-binding
2402	Q25472	Actin, muscle-type	148	42521	5.12	41	+ - +	Structure, Cell motility, muscle contraction
2403	Q5E9B5	Actin, gamma-enteric smooth muscle	87	42249	5.31	25	- + -	Mesenchyme migration, muscle contraction
2405	P08537	Tubulin alpha chain	125	50532	4.96	27	+ + -	Microtubule-based process
2501	Q2KJD0	Tubulin beta-5 chain	92	50095	4.78	18	+ + -	Spindle assembly
3404	P02542	Desmin	78	53304	5.45	37	+ - +	Intermediate filament organization, cytoskeletal organization
4706	Q28046	Adseverin	69	80984	5.67	11	+ + -	Actin capping, regulation of chondrocyte differentiation
3706	Q08043	Alpha-actinin-3	76	103917	5.37	8	+ + -	Bundling protein, muscle filament sliding, calcium-metal binding
Energy metabolism								
3501	Q9PTY0	ATP synthase subunit beta, mitochondrial	92	56650	5.05	24	- + -	ATP synthesis, transport, lipid metabolic process

7210	O74988	Cytochrome c oxidase polypeptide 5, mitochondrial	71	20836	9.61	34	+ - -	Hydrogen ion transmembrane transport, cytochrome c to oxygen
5511	P51913	Alpha-enolase	114	47617	6.17	39	+ - +	Gluconeogenesis, glycolytic process
7406	P14223	Fructose-bisphosphate aldolase	72	40479	8.33	31	+ - -	Glycolysis, carbohydrate degradation
7401	O77760	Sterol O-acyltransferase 1	68	65198	9.08	7	+ + -	Cholesterol, lipid, steroid and sterol metabolism
Nutrient metabolism and transport								
5102	P11757	Hemoglobin subunit alpha	66	15244	7.98	50	- + +	Oxygen transport, Transport
5305	P46410	Glutamine synthetase	68	42630	6.28	17	+ - -	Glutamine biosynthetic process, essential for proliferation of fetal skin fibroblasts
5410	P10760	Adenosylhomocysteinase	79	48020	6.07	25	+ - +	One-carbon metabolism, amino-acid biosynthesis
6508	Q06087	Tyrosine decarboxylase 3	81	49099	7.67	24	+ - +	Cellular amino acid metabolic process
3302	P53824	Probable pyridoxal 5'-phosphate synthase subunit SNZ2	67	32298	5.4	18	+ + -	pyridoxal phosphate biosynthetic process, pyridoxine biosynthetic process
Cellular homeostasis								
5211	P91254	Probable glutathione S-transferase 8	69	23475	6.16	34	+ + -	Transferase, conjugation of reduced glutathione
Protein synthesis, processing, regulation								
7604	A4H3Z2	Pescadillo homolog	69	77503	9.58	12	+ - -	Ribosome biogenesis, rRNA processing
8204	Q9SGA6	40S ribosomal protein S19-1	72	15875	10.09	44	+ - -	Ribosomal small subunit assembly, translation
8307	Q9P015	39S ribosomal protein L15, mitochondrial	69	33570	10.02	24	+ - -	Mitochondrial translational elongation and termination
Protein transport								

7205	P57745	Mitochondrial import inner membrane translocase subunit Tim9 O	73	10419	8.42	25	+ - -	Protein transport, translocation, chaperone
				Signaling pathways				
7305	Q62923	Prepronociceptin	72	20864	8.49	25	+ + -	Neuronal differentiation and development, neuropeptide signaling parthway
2208	Q5ZKC9	14-3-3 protein zeta	66	27929	4.73	25	+ - -	Protein targeting, regulation, signaling pathways
				Embryonic development				
7304	Q801G2	Membrane progestin receptor alpha-B	73	42097	7.26	24	+ + -	Steroid signaling, differentiation, oogenesis
1702	Q92834	X-linked retinitis pigmentosa GTPase regulator	66	114342	4.79	7	- + -	Sensory transduction, vision, cilium biogenesis/degradation
7506	Q13105	Zinc finger protein 17	67	79720	8.81	11	+ - -	Early embryonic development, ectoderm development, transcription
				Proteins folding				
2504	P07823	78 kDa glucose-regulated protein	133	72505	5.07	24	+ - -	Positive regulation of cell migration, chaperone
3506	O73885	Heat shock cognate 71 kDa protein	84	71011	5.47	18	+ - +	regulation of apoptosis during embryonic development, chaperone
2302	Q9STN7	Protein BOBBER 2	72	33670	5.19	21	+ - -	Multicellular organism development, chaperone
				Protein modification				
7404	Q92182	Beta-galactoside alpha-2,6-sialyltransferase 1	68	47818	8.96	13	+ - +	Protein sialylation
				Others				
2901	Q5RBK0	Gypsy retrotransposon integrase-like protein 1	66	186718	5.07	13	- - +	DNA integration
7507	Q19473	Serpentine receptor class delta-51	72	39061	9.37	10	+ - -	Integral component of membrane

[a] UniProt serial number.

[b] Protein score higher than 65 were considered statistically significant (P<0.05).

[c] Sequence percent coverage.

[d] Indicates protein differential expression between gastrula (early gestation), 34 (mid gestation) and 39 (late gestation) developmental stages respectively. Protein expression: (+) protein upregulated, (-) protein downregulated.

Table 3. Reproducibility of 2-DE gel protein quantitation from gel to gel and gestation groups in *Mabuya* sp. This inter-experiment compares gels from 3 different samples from the same gestation group, prepared on different occasions.

Gestation	Spots matched[a]	Match rate%[b]	Exp I vs II[c]	Exp I vs III[d]	Exp II vs III[e]	CV[f]
Early	318	89	0.849	0.874	0.842	50.98
Mid	45	92	0.714	0.721	0.72	40.94
Late	34	83	0.732	0.731	0.702	40.93

[a] Spots completely matched in the three 2-DE gels per gestation group (PDQuest software v.8.0)

[b] Percentage of matched spots relative to the total number of spots on the gel

[c, d, e] Correlation coefficient between biological replicates.

[f] Mean coefficient of variation inter-groups

We searched for known and predicted interactions for the differentially expressed proteins in the placenta identified by MALDI-TOF in the STRING protein-protein interaction database and constructed a protein-protein interaction network (Figure 7), e.g. one pathway association observed within these groups of proteins is the linkage between actin (ACTB, ACTG2, ACTA1) and tubulin (TUBA1A), (their interaction is experimentally determined, pink line) and its co-expression (black line) with ATP synthase subunit beta (ATP5B). This co-expression with ATP synthase would be related to energy synthesis and supply to meet the requirements of the several processes in which cytoskeletal proteins are involved as cell motility, cell cycle and cell structure among others (Figure 7).

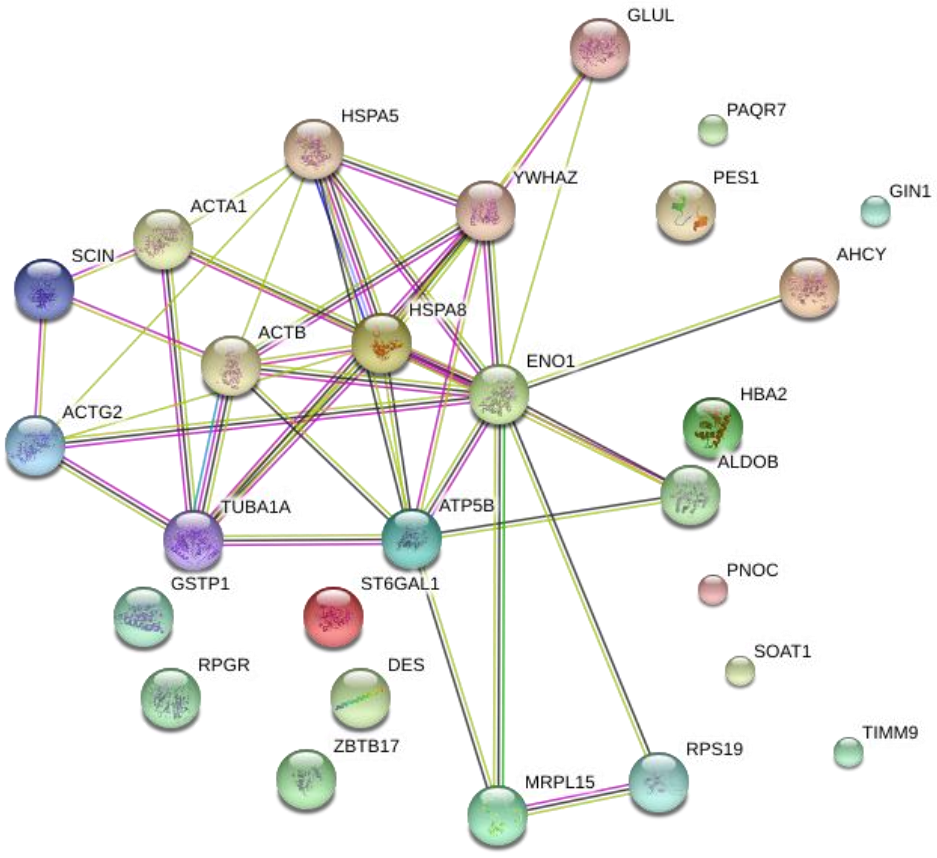

Figure 7. Protein-protein interactions for the differentially expressed in *Mabuya* sp placental proteins during gestation and identified by MALDI-TOF were visualized through proteomic pathway software STRING v10.0. In the predicted network,

proteins are represented as nodes. Small nodes are proteins of unknown 3D structure, while large nodes are proteins with 3D predicted or known structure. Colors of the lines connecting the nodes represent different evidence types for protein linkage. Known interactions are represented with pink (experimentally determined) and light blue (curated databases). Predicted interactions are represented with blue (co-occurrence) and green (neighborhood). Textmining is represented with light green lines and co-expression is represented with black lines.

4. DISCUSSION

2-DE coupled with MS is one of the most classical quantitative proteomics techniques. Quantitative proteomics investigate physiology at the molecular level by measuring relative differences in protein expression between samples under different experimental conditions. A major obstacle for reliably in the determination of quantitative changes in protein expression is to overcome error imposed by technical and biological variations. Although all experiments are subject to several degrees of variation, including technical “procedural noise” and the inherent heterogeneity of the biological samples, not all experiments can be conducted using tightly controlled model system, since there might be some limitations (Molloy *et al*. 2003). In this scenario, we found some limitations with the protein quantity required for 2-DE e.g. specimens can be very difficult to capture, sometimes collecting them is more a matter of good fortune than design.

With this electrophoretic technique we can access a fraction of all proteins available in the proteome. The spots obtained in the experiments were representative of the physiological events found in the ovarian samples in terms of concentration differences. Currently, it is not technically possible to access all proteins expressed in a tissue only by one technique, e.g. because proteins are expressed at different concentration ranges, with different biochemical properties (pI, MW, etc) or because of post-transcriptional mechanisms that control rates of synthesis and half-life of

proteins, which have suggested that the mRNA level of a particular gene might not accurately reflect the amount of the corresponding protein expressed in the same cell.

For this reason, it should be highlighted that these proteins differentially expressed throughout gestation gave us a preliminary idea of the genes fraction being expressed at any one time, since the variety of gene expression profiles characteristic of different cell types arise because these cells have distinct sets of transcription regulators, e.g. some regulators work to increase transcription, whereas others prevent or suppress it (Cox *et al*. 2005). Therefore, an integrated approach between “omics” e.g. genomic, transcriptomic, metabolomic and proteomic is proven to be a powerful approach in characterizing the changes in biological processes (Tian 2004).

Here, we present an insight into the proteins expressed during gestation in the first report of the reproductive tissues (ovary and placenta) in *Mabuya* sp, (discussed immediately) in different stages of gestation.

4.1 BIOLOGICAL RHYTHM

4.1.1 Ovarian tissues

Circadian clock-controlled protein is a component of the circadian rhythm and is involved in multicellular organism reproduction (Lorenz, Hall, & Rosbash, 1989). Most circadian clocks genes are being studied in the cells of the ovary, uterus and oviduct in birds and mammals where they may play a role regulating the timing of oviposition-ovulation respectively (Sellix & Menaker, 2010). Circadian clock-controlled protein was highly expressed in mid and late gestation in *Mabuya*’s ovary, and probably is regulating the timing of ovulation, a critical step to the success of reproduction.

4.2 FOLLICULAR DEVELOPMENT AND REGULATION

4.2.1 Ovarian tissues

Lissencephaly-1 (Lis1) is a conserved protein highly expressed in *Mabuya* sp ovary during mid and late gestation period. Lis1 is required for several dynein and microtubule-dependent processes (Dix *et al*., 2013). Lis1 also regulates bone morphogenetic protein (BMP) signaling and E-cadherin-mediated adhesion between germline stem cell (GSC) (Chen *et al*., 2010). Most of the presumptive ovarian roles of BMPs remain unexplored in reptiles and birds, except in hen and mammals where play an important role in the regulation of ovarian function, evidenced by the ability of BMPs to control folliculogenesis, granulosa cell proliferation and cytodifferentiation, as well as oocyte development (Persani, Rossetti, Di pasquale, Cacciatore, & Fabre, 2014).

Nuclear autoantigen SP100 is highly expressed in mid and late gestation in *Mabuya* sp. The nuclear autoantigen SP100 (speckled protein 100) is reported to control cellular gene expression, cell growth and differentiation (Held-Feindt, Hattermann, Knerlich-Lukoschus, Mehdorn, & Mentlein, 2011). Also, through the regulation of ETS1, it may play a role in angiogenesis, controlling endothelial cell motility and cell invasion (Wasylyk, Schlumberger, Criqui-Filipe, & Wasylyk, 2002). In mammals, regulation of ETS1 by nuclear SP100 enables the follicle and/or corpus luteum (CL) to receive the required supply of nutrients, oxygen and hormonal support as well as facilitating the release of steroids (Robinson *et al*., 2009).

In this sense, upregulation of nuclear autoantigen SP100 in *Mabuya*'s sp ovary in mid and late gestation, probably helps to supply the molecules that are necessary to the follicular development through the small blood and lymphatic capillaries present in the separation between the theca interna and externa, and in the corpus luteum when luteolysis is progressing (some fibers, blood vessels and fibroblast progressively invade the luteal tissue) (Gómez & Ramírez-Pinilla, 2004).

4.3. ENERGY METABOLISM

4.3.1 Ovarian tissues

Long chain fatty acid CoA ligase (ACSL) activates fatty acids from exogenous sources for the synthesis of triacylglycerol destined for intracellular storage (Kang *et al*., 1997). ACSL-5 upregulation in mid and advanced gestation of *Mabuya* sp may be associated with energy supply for oocyte maturation, vitellogenesis and ovulation, since the majority of fat bodies and other peripheral lipid stores, such triglycerides are often built up prior to vitellogenesis in most reptiles (e.g. in *Mabuya* sp fatty accumulation in gestation occurs at the end of gestation) (Gómez & Ramírez-Pinilla, 2004), birds (Price, 2016) and mammals (Kang *et al*., 1997).

4.4 CELL MORPHOLOGY AND MOTILITY

4.4.1 Placental tissues

Highly conserved cytoskeletal proteins such tubulin (α and β-5 chain), actin (cytoplasmic and alpha-skeletal), desmin, adseverin (Ads) and α-actinin-3 (actin binding protein) were upregulated in placental tissues during early and mid gestation. In early gestation, this upregulation is related to amniote morphogenetic re-arrangements when the zygote is under cleavage and cellular differentiation e.g. upregulation of cytoskeltal proteins had been described in chicken (Toyoizumi, Shiokawa, & Takeuchi, 1991) and mammalian (Sparn, Lieder-Ochs, & Franke, 1994) gastrulation, and principally in reptiles, where a bi-modal strategy of gastrulation (involution and ingression) has been sugested (Stower *et al*., 2015).

Physyological high expression of cytoskeletal proteins in *Mabuya* sp placenta in mid gestation (actin alpha-skeletal, gamma actin, ads and α-actinin-3) might be related to the increasing demands of the growing embryo. In *Mabuya* sp this process require destruction, growth and change of the uterine luminal epithelium of the placentoma

(which forms a synsytium), the syncytial cells are limited to the folding zone during mid gestation, later at an advanced gestation the syncytial cells are completely extended in the placentome, the cellular change between the syncytial zone of the uterus in the placentome and the uterine epithelium of the paraplacentome is very drastic (Jerez & Ramírez-Pinilla, 2001) and reasoning require the high expression of cytoskeletal proteins, which are reported in this work. In *C. ocellatus* the increased size during embryonic development, also improves nutrient interchange during gestation by maximizing the surface area of the maternal–embryonic interface (Brandley *et al*., 2012).

The high expression of actin muscle type and desmin (Table 2), proteins regulating muscle contraction are found in an advanced gestation period. In mammals and *C. ocellatus* late pregnancy, there was a similar upregulation of cytoskeletal genes (β-actin and gesolin, a powerful acting regulator) (Brandley *et al*., 2012). Regulation of actin cytoskeleton is the most representative biological pathway in which human placental proteins are predicted to be involved (Lee *et al*., 2013), e.g. expression of α-actinin and Ads in mammalian placenta are related to the expasion of uteroplacental tissues, and muscle actin is related to muscle contraction when the labour is near (Nanaev *et al*., 2000).

4.5 ENERGY METABOLISM

4.5.1 Placental tissues

In amniotes, glucose is one of the major nutrients for the growing embryos in reptiles, birds (Scanes & Braun, 2013) and mammals, where the fetus has a very low capacity for glucose production, so fetal mammalian glucose levels are primarily controlled through placental transport from the maternal circulation (Novakovic, Gordon, Robinson, Desoye, & Saffery, 2013). Similarly, there was an upregulation of enzymes implicated in glucose metabolism (glycolytic and gluconeogenic pathways)

in *Mabuya* sp at gastrulation. Alpha-enolase and fructose-bisphosphate aldolase (FBP) upregulation (Table 2) seemed vital for energy supply and embryo development under anaerobic conditions, these enzymes are present but downregulated at mid-gestation when the efficient and specialized allantoplacenta forms to nourish the embryo and gas exchange, demonstrating carbohydrate transference by the allantoplacenta during all the gestation but particularly in the beginning of it (Ramírez-Pinilla *et al*., 2011; Wooding, Ramirez-Pinilla, & Forhead, 2010).

Sterol O-acyltransferase 1, an enzyme that catalyzes the formation of fatty acid-cholesterol esters (CE), and activated by the presence of cholesterol is highly upregulated at mid gestation. This high expression in *Mabuya* sp suggest that lipid metabolism is the central pathway once the allantoplacenta has developed its greatest complexity (Ramírez-Pinilla *et al*., 2011), cholesterol also serve as a reservoir for producing steroid hormones, needed for embryonic development, maintenance and growth of the mammalian embryo (Chang, Li, Chang, & Urano, 2009).

Physiological studies have demonstrated that lipids represent the primary source of energy in oviparous reptiles (produced within egg and supporting the metabolic costs of growth and maintenance of the developing embryo) (Price, 2016) and in numerous squamate viviparous species with a relatively complex placenta, where there is a net uptake of lipids across the placenta (Griffith, Ujvari, Belov, & Thompson, 2013; Ramírez-Pinilla *et al*., 2011; Speake, Herbert, & Thompson, 2004; Thompson & Speake, 2006).

4.6 NUTRIENT METABOLISM AND TRANSPORT

4.6.1 Placental tissues

Viviparous snakes with a simple placenta, *Agkistrodon contortrix* (Viperidae), *Boa constrictor* (Boidae), *Nerodia sipedon* (Colubridae: Natricinae) and *Thamnophis sirtalis* (Colubridae: Natricinae) are capable of transporting diet-derived amino acids to their offspring during gestation, possibly via placentation (Van Dyke, Beaupre, & Kreider, 2012). It seems that in the last third of pregnancy in viviparous squamate lizards is particularly crucial the provision of nutrients and ions (Itonaga, Wapstra, & Jones, 2012): in *C. ocellatus* late pregnancy period, inorganic and organic molecular transport of glucose, amino acids, peptides, fatty acids, and carboxylic acids is highly being carried out (Brandley *et al*., 2012).

In *Mabuya* sp there is also a significant net uptake of inorganic and organic matter, this transplacental nutrient transfer begins slowly during early gestation (gastrula and neurula/pharyngula), accelerates in limb bud stages and its maximum in advanced gestation and preparturition stages (Wooding *et al*., 2010). The upregulation of adenosylhomocysteinase and tyrosine decarboxylase 3 (enzymes implicated in the amino acid biosynthesis) at the end of gestation in *Mabuya* sp (Table 2), could be due to the high amount of nutrient transference in this period (Ramírez-Pinilla *et al*., 2011).

The active form of vitamin B6 (pyridoxal 5-phosphate, PLP), is de novo synthesized by pyridoxal 5'-phosphate synthase and from substrates like, glyceraldehyde 3-phosphate and ammonia (hydrolyzed from glutamine by a complexed glutaminase) (Qaidi, Yang, Zhang, Metzger, & Bai, 2013). PLP is an essential cofactor for numerous metabolic enzymes, in mammalian gestation is required for fetal and placental development. The placenta itself needs nutrients to growth earlier than the

fetus, especially during early phases when increases in weight fastest (Baker, Frank, Deangelis, Feingold, & Kaminetzky, 1981).
In human's gestation, vitamin B6 is provided to the fetus exclusively from the mother and depends on simple facilitated diffusion and/or active transport in sufficient amounts to meet the need of the growing embryo (Schenker, Johnson, Mahuren, Henderson, & Coburn, 1992).

Probable pyridoxal 5'-phosphate synthase subunit SNZ2 was high expressed in early and mid-gestation of *Mabuya* sp. Vitamin B6 possibly could be transferred by the mature placenta in mid gestation. Previous studies in *Mabuya* sp also have suggested that vitamin (vitamin E, α-tocopherol) is transferred by the placenta, since there was detected in some of the embryonic stages, embryos and neonates (Ramírez-Pinilla *et al*., 2011). Similarly, there is a strong evidence that macromolecules are transported across uterine epithelium in the viviparous skinks Pseudemoia entrecasteauxii and P. spenceri and later be absorbed by the developing fetus (Biazik, Thompson, & Murphy, 2009; Griffith *et al*., 2013).

On the other hand, one metabolic protein upregulated in early gestation of *Mabuya* sp is glutamine synthetase (GS). GS removes ammonia or glutamate in tissues (Filho, Costa, Wu, McKeever, & Watford, 2009), is essential for cell proliferation and synthesis of glutamine de novo (Vermeulen *et al*., 2008). In most mammalian species, the developing fetus utilizes large amounts of glutamine for nutrition (necessary for the metabolism of nucleic acids and sugars (He, Hakvoort, Vermeulen, Lamers, & Van Roon, 2007), glutamine is synthesized de novo in the placenta and derived from maternal circulation (Filho *et al*., 2009; He *et al*., 2007; Self, 2004). GS gene was also identified in the late pregnancy of the viviparous skink *C. ocellatus* (Brandley *et al*., 2012). Although GS is downregulated in *Mabuya* sp mid and late gestation compared to early gestation, its expression in placental tissues probably illustrate substancial capacity for glutamine synthesis and remotion of metabolic waste in the early placenta as ammonia. GS upregulation has been

suggested as a critical step to maintaining both reptilian and mammalian pregnancies (Brandley *et al*., 2012).

4.7 CELLULAR HOMEOSTASIS

4.7.1 Placental tissues

Glutathione S-transferase is present in mammalian fetus and placenta. In early mammalian embryonic development regulates trophectoderm (TE) cell lines (Raijmakers, Steegers, & Peters, 2001; Talbot, Powell, Caperna, & Garrett, 2010). TE cells are the first to differentiate, their differentiation is necessary for pregnancy recognition, implantation and formation of the extra-embryonic tissues (Mohapatra *et al*., 2015). Expression of glutathione S-transferase (GT) in human placental tissues (Sekijima *et al*., 1999) is important in intracellular binding and transport in detoxification processes, since GTs catalyzes the conjugation of glutathione to toxic compounds, resulting in more water-soluble and less biologically active products that may be easily excreted (Raijmakers *et al*., 2001). Glutathione peroxidases 1,3-5 are similarly upregulated in *C. ocellatus* pregnancy, these enzymes function to scavenge reactive oxygen species that are the by-product of oxidative metabolism (Brandley *et al*., 2012).

We found upregulation of probable glutathione S-transferase 8 (Gts8) during *Mabuya* sp early and mid-gestation. The high expression possibly is related to cellular redox homeostasis of both uterus and extraembryonic membranes during gestation and development, especially in mid gestation when nutrient exchange and waste products by the placentome (Leal & Ramírez-Pinilla, 2008) need to be removed, limiting oxidative damage and other stress responses.

4.8 PROTEINS SYNTHESIS, PROCESSING AND REGULATION

4.8.1 Placental tissues

Pescadillo homolog protein, 40S ribosomal protein S-19-1 and 30 ribosomal protein L15 (mitochondrial) showed a high expression in early gestation and lower expression in placental tissues in mid and late gestation in *Mabuya* sp, in consequence, these proteins seemed to be required to development of the embryo. Pescadillo (pes), mitochondrial 39S ribosomal protein L15 and 40S ribosomal protein S19-1 are involved in ribosome biogenesis, a fundamental process that ensures the cell's protein synthesis to meet the demand of growth, proliferation, and adaptation of the embryo (Flygare *et al*., 2007; Matsson *et al*., 2004). Pes is widely expressed in early zebrafish embryogenesis (Allende *et al*., 1996) and growth of mouse embryos (Maiorana, Tu, Cheng, & Baserga, 2004), is required in nucleolar assemble and mammalian cell proliferation (Lerch-Gaggl *et al*., 2002),

4.9 PROTEIN MODIFICATION

4.9.1 Placental tissues

Beta-galactoside alpha-2,6-sialyltransferase 1 is upregulated in early and late gestation in *Mabuya* sp. β-galactoside α-2,6-sialytransferase has been found in both embryos of chicken (Kurosawa *et al*., 1994) and human placenta, it mediates the attachment of sialic acid to glycoprotein sugar chains (Grundmann, Nerlich, Rein, & Zettlmeissl, 1990), e.g. is involved in the construction of complex N-linked oligosaccharides, which function in cell recognition, cell binding and regulation of the protein conformation (Rabouille *et al*., 1995). These steps are part of the protein glycosylation pathway, an ubiquitous post-translational modification that influences almost all aspects of normal cell biology (Moremen, Tiemeyer, & Nairn, 2012).

4.10 SIGNALING

4.10.1 Placental tissues

Prepronociceptin was high expressed in gastrula and mid gestation in placental tissues of *Mabuya* sp. Nociceptin is a neurotransmitter and a neuroendocrine hormone derived from the large precursor prepronociceptin. It is involved in developmental signaling during gestation (Mollereau *et al*., 1996), is also required for the formation of anterior placode (the transient ectodermal thickening epithelium in the embryonic head, that gives rise to neurons and other structures of the sensory nervous system, Graham & Shimeld 2013) in Xenopus, chicken and zebrafish during early development (Lleras-Forero *et al*., 2013).

The 14-3-3 protein zeta is highly upregulated early in *Mabuya* sp gestation, late in placental tissues its expression decreased, suggesting an important role in the embryo. 14-3-3 protein zeta is implicated in regulation of a large spectrum of both general and specialized signaling pathways, in human placenta binds and modulates a large numer of partners, usually by recognition of a phosphoserine or phosphothreonine motif (Zupan, Steffens, Berry, Landt, & Gross, 1992). 14-3-3 protein zeta is also essential for cell fate specification in Drosophila embryos and during embryogenesis in Homo sapiens (Darling, Yingling, & Wynshaw-Boris, 2005).

4.11.1 EMBRYONIC DEVELOPMENT

-Placental tissues

Zinc finger protein 17 is high expressed at gastrula developmental stage, later its expression decreased in mid and late gestation in *Mabuya* sp placenta, seemed to be more related to embryonic development. Zinc finger protein 17 is a transcription factor shown to be vital for early embryonic development: it is essential for ectoderm

development, deuterostomic gastrulation and early lymphocyte development in chicken and mammals, where it prevent apoptosis in lymphoid precursors (Sefton, Sánchez, & Nieto, 1998).

Progesterone (Pg) is critical for the establishment and the maintenance of pregnancy, both by its endocrine and immunological effects during gestation in vertebrates (Arck, Hansen, Mulac Jericevic, Piccinni, & Szekeres-Bartho, 2007; Tuckey, 2005). In addition to their well-documented genomic effects, Pg also stimulates a “non-genomic” rapid action on cell-signaling pathways through membrane progesterone receptors (mPRs) (Moussatche & Lyons, 2012). Recently, homologues proteins of the fish membrane progestin receptors were identified in human, mouse, pig, Xenopus, zebrafish and Fugu, and were classified as mPRα, β and γ (Zhu, Bond, & Thomas, 2003).

The mPRα is widely expressed in different vertebrate species and reproductive tissues (ovary, testis, uterus and placenta), suggesting its multiple physiological functions (Hossain, Oshima, Hirose, Wang, & Tokumoto, 2015), e.g. mPRα mediates non-genomic actions of progestins including, the secretion of gonadotropin-releasing hormone (GnRH), maturation of oocytes in fish, immune modulation, vasodilation, transportation and relaxation of the uterine myometrium during mammalian pregnancy (Dressing, Goldberg, Charles, Schwertfeger, & Lange, 2011; Thomas, 2008). mPRα is mostly expressed in the mammalian placenta and is significantly upregulated in the endometrium throughout gestation, except near to the end of gestation where its expression decreased, this withdrawal is associated with a marked reduction in myometrial quiescence due to labour (Fernandes, 2005).

Very similar to the reported in the viviparous skink *C. ocellatus* (significant downregulation of progesterone receptor and estrogen receptor in uterus during late gestation) (Brandley *et al*., 2012) and in mammals, there was a high expression of

membrane progestin receptor alpha-B in early and mid *Mabuya*'s sp gestation. This expression may be possibly a non-classical function of Pg, and provides evidence about expression of Pg in the extra-embryonic membranes, an important step to decrease the myometrial contractility in this period of pregnancy. Later at the end of gestation in *Mabuya* sp, expression of membrane progestin receptor α-B decreased, probably to facilitate myometrial contraction.

X-linked retinitis pigmentosa GTPase regulator (RPGR) is highly expressed in mid gestation of *Mabuya* sp. RPGR acts in ciliogenesis and transport of primary cilia, microtubule-based organelles required for the responses to specific intercellular signals (Bangs, Schrode, Hadjantonakis, & Anderson, 2015). RPG has been detected in mouse and human placenta-oviduct where is involved in numerous activities such as cell signaling, processing of developmental signals, and directing the flow of fluids such as mucus over and around the cells (Bangs *et al*., 2015; Kirschner *et al*., 1999).

4.12 PROTEIN FOLDINGS

4.12.1 Placental tissues

Heat shock proteins (HSPs) are molecular chaperone with cytoprotective functions found in all organisms from yeast to humans, involved in transport, folding, assembly and degradation of other proteins (Åkerfelt, Morimoto, & Sistonen, 2010; Molvarec *et al*., 2010). Heat shock 78 kDa glucose-regulated protein, heat shock cognate 71 kDa protein (Hsc70) and protein bobber 2 (small heat shock protein) are proteins upregulated in uterus and embryo during early development of *Mabuya* sp (Table 2). Varied HSPs expression appeared to be very essential in critical steps of early embryonic development, regulating processes as cell movements, proliferation, morphogenesis and apoptosis (Rupik, Jasik, Bembenek, & Widłak, 2011).

Hsc70 is also upregulated at the end of *Mabuya* sp gestation. In mammals, gestation brings oxidative stress due to the extensive cell proliferation and the high metabolic activity, particularly at the end of pregnancy as a feature of labor (Abdulsid, Hanretty, & Lyall, 2013; Tedeschi *et al*., 2016), Hsc70 possibly is high expressed to regulate apoptosis that brings oxidative stress (De la Rosa *et al*., 1998)

5. CONCLUSIONS

In this study, we found the preliminary identification by 2-DE and MALDI-TOF technology of proteins with different expression profiles related to *Mabuya* sp useful to understand the physiological development pregnancy mechanism in viviparous reptiles. A total of 14 and 37 proteins were identified in ovarian and placental tissues respectively. Additionally, most of proteins expressed are highly conserved and might play a similar role to those proteins regulating gestation and growing in the embryo in ammniotes.

Accurately identifying the technical steps associated with the variability would provide valuable information for the improvement and design of future processing data. Further “omics” analysis should be performing in order to complement the understanding throughout the *Mabuya*’s sp. We also must highlight the current proteomics approaches which differ in the types of instrument used, their performance profiles, the manner in which they interface with biological research strategies, and their reliance on and use of prior information.

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7. REFERENCES

Abdulsid, A., Hanretty, K., & Lyall, F. (2013). Heat shock protein 70 expression is spatially distributed in human placenta and selectively upregulated during labor and preeclampsia. *PLoS ONE*, *8*(1), e54540.

Åkerfelt, M., Morimoto, R. I., & Sistonen, L. (2010). Heat shock factors: integrators of cell stress, development and lifespan. *Nature Reviews Molecular Cell Biology*, *11*(8), 545–555.

Allende, M. L., Amsterdam, A., Becker, T., Kawakami, K., Gaiano, N., & Hopkins, N. (1996). Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development. *Genes & Development*, *10*(24), 3141–3155.

Arck, P., Hansen, P. J., Mulac Jericevic, B., Piccinni, M.-P., & Szekeres-Bartho, J. (2007). Progesterone during pregnancy: endocrine immune cross talk in Mammalian species and the role of stress. *American Journal of Reproductive Immunology*, *58*(3), 268–279.

Baker, H., Frank, O., Deangelis, B., Feingold, S., & Kaminetzky, H. (1981). Role of placenta in maternal-fetal vitamin transfer in humans. *American Journal of Obstetrics and Gynecology*, *141*(7), 792–796.

Bangs, F. K., Schrode, N., Hadjantonakis, A., & Anderson, K. (2015). Lineage specificity of primary cilia in the mouse embryo. *Nature Cell Biology*, *17*(2), 113–122.

Beranova-Giorgianni, S. (2003). Proteome analysis by two-dimensional gel electrophoresis and mass spectrometry: strengths and limitations. *TrAC Trends*

in Analytical Chemistry, *22*(5), 273–281.

Biazik, J. M., Thompson, M. B., & Murphy, C. R. (2009). Lysosomal and alkaline phosphatase activity indicate macromolecule transport across the uterine epithelium in two viviparous skinks with complex placenta. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *312B*(8), 817–826.

Blackburn, D. G. (2000). Classification of the Reproductive Patterns of Amniotes. *Herpetological Monographs*, *14*(2000), 371. http://doi.org/10.2307/1467051

Blackburn, D. G. (2006). Squamate Reptiles As Model Organisms for the Evolution of Viviparity. *Herpetological Monographs*, *20*(1), 131. http://doi.org/10.1655/0733-1347(2007)20[131:SRAMOF]2.0.CO;2

Blackburn, D. G., & Flemming, A. F. (2009). Morphology, development, and evolution of fetal membranes and placentation in squamate reptiles. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *312B*(6), 579–589. http://doi.org/10.1002/jez.b.21234

Blackburn, D. G., & Vitt, L. J. (1992). Reproduction in viviparous south American lizards of the genus *Mabuya*. In *Reproductive Biology of South American Vertebrates* (pp. 150–164). New York, NY: Springer New York. http://doi.org/10.1007/978-1-4612-2866-0_11

Blackburn, D. G., & Vitt, L. J. (2002). Specializations of the chorioallantoic placenta in the Brazilian scincid lizard, *Mabuya* heathi: A new placental morphotype for reptiles. *Journal of Morphology*, *254*(2), 121–131. http://doi.org/10.1002/jmor.10005

Blackburn, D. G., Vitt, L. J., & Beuchat, C. a. (1984). Eutherian-like reproductive specializations in a viviparous reptile. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(15), 4860–4863. http://doi.org/10.1073/pnas.81.15.4860

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248–254. http://doi.org/10.1016/0003-

2697(76)90527-3

Brandley, M. C., Young, R. L., Warren, D. L., Thompson, M. B., & Wagner, G. P. (2012). Uterine gene expression in the live-bearing lizard, *Chalcides ocellatus*, reveals convergence of Squamate reptile and mammalian pregnancy mechanisms. *Genome Biology and Evolution*, *4*(3), 394–411. http://doi.org/10.1093/gbe/evs013

Centlow, M., Hansson, S. R., & Welinder, C. (2010). Differential proteome analysis of the preeclamptic placenta using optimized protein extraction. *Journal of Biomedicine and Biotechnology*, *2010*, 1–9. http://doi.org/10.1155/2010/458748

Chang, T., Li, B., Chang, C. C. Y., & Urano, Y. (2009). Acyl-coenzyme A : cholesterol acyltransferases. *American Journal of Physiology. Endocrinology and Metabolism*, *297*, 1–9. http://doi.org/10.1152/ajpendo.90926.2008.

Chen, S., Kaneko, S., Ma, X., Chen, X., Ip, Y. T., Xu, L., & Xie, T. (2010). Lissencephaly-1 controls germline stem cell self-renewal through modulating bone morphogenetic protein signaling and niche adhesion. *Proceedings of the National Academy of Sciences*, *107*(46), 19939–19944. http://doi.org/10.1073/pnas.1008606107

Darling, D. L., Yingling, J., & Wynshaw-Boris, A. (2005). Role of 14–3–3 Proteins in Eukaryotic Signaling and Development. In *Current Topics in Developmental Biology* (Vol. 68, pp. 281–315). http://doi.org/10.1016/S0070-2153(05)68010-6

De la Rosa, E. J., Vega-Nunez, E., Morales, A. V., Serna, J., Rubio, E., & de Pablo, F. (1998). Modulation of the chaperone heat shock cognate 70 by embryonic (pro)insulin correlates with prevention of apoptosis. *Proceedings of the National Academy of Sciences*, *95*(17), 9950–9955. http://doi.org/10.1073/pnas.95.17.9950

Dix, C. I., Soundararajan, H. C., Dzhindzhev, N. S., Begum, F., Suter, B., Ohkura, H., … Bullock, S. L. (2013). Lissencephaly-1 promotes the recruitment of dynein and dynactin to transported mRNAs. *Journal of Cell Biology*, *202*(3), 479–494. http://doi.org/10.1083/jcb.201211052

Dressing, G. E., Goldberg, J. E., Charles, N. J., Schwertfeger, K. L., & Lange, C. A.

(2011). Membrane progesterone receptor expression in mammalian tissues: A review of regulation and physiological implications. *Steroids*, *76*(1-2), 11–17. http://doi.org/10.1016/j.steroids.2010.09.006

Dufaure, J. P., & Hubert, J. (1961). Table de dévelopment du lézard vivipare, Lacerta (Zootoca) vivipara jacquin. *Archives D Anatomie Microscopique Et De Morphologie Experimentale*, *50*(3), 309–328.

Fernandes, M. S. (2005). Regulated expression of putative membrane progestin receptor homologues in human endometrium and gestational tissues. *Journal of Endocrinology*, *187*(1), 89–101. http://doi.org/10.1677/joe.1.06242

Filho, M., Costa, H. E., Wu, G., McKeever, K. H., & Watford, M. (2009). Equine placenta expresses glutamine synthetase. *Veterinary Research Communications*, *33*(2), 175–182. http://doi.org/10.1007/s11259-008-9167-2

Flygare, J., Aspesi, A., Bailey, J. C., Miyake, K., Caffrey, J. M., Karlsson, S., & Ellis, S. R. (2007). Human RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. *Blood*, *109*(3), 980–986. http://doi.org/10.1182/blood-2006-07-038232

Fox, S. L., & Guillette, L. J. (1987). Luteal morphology, atresia, and plasma progesterone concentrations during the reproductive cycle of two oviparous lizards, Crotaphytus collaris and Eumeces obsoletus. *American Journal of Anatomy*, *179*(4), 324–332. http://doi.org/10.1002/aja.1001790403

Gómez, D., & Ramírez-Pinilla, M. P. (2004). Ovarian histology of the placentotrophic *Mabuya* mabouya (Squamata, Scincidae). *Journal of Morphology*, *259*(1), 90–105. http://doi.org/10.1002/jmor.10170

Görg, A., Weiss, W., & Dunn, M. J. (2004). Current two-dimensional electrophoresis technology for proteomics. *Proteomics*, *4*(12), 3665–3685. http://doi.org/10.1002/pmic.200401031

Graham, A., & Shimeld, S. M. (2013). The origin and evolution of the ectodermal placodes. *Journal of Anatomy*, *222*(1), 32–40. http://doi.org/10.1111/j.1469-7580.2012.01506.x

Griffith, O. W., Brandley, M. C., Belov, K., & Thompson, M. B. (2016). Allelic expression of mammalian imprinted genes in a matrotrophic lizard, Pseudemoia entrecasteauxii. *Development Genes and Evolution*, *226*(2), 79–85. http://doi.org/10.1007/s00427-016-0531-x

Griffith, O. W., Brandley, M. C., Whittington, C. M., Belov, K., & Thompson, M. B. (2016). Comparative genomics of hormonal signaling in the chorioallantoic membrane of oviparous and viviparous amniotes. *General and Comparative Endocrinology*. http://doi.org/10.1016/j.ygcen.2016.04.017

Griffith, O. W., Ujvari, B., Belov, K., & Thompson, M. B. (2013). Placental lipoprotein lipase (LPL) gene expression in a placentotrophic lizard, Pseudemoia entrecasteauxii. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *320*(7), 465–470. http://doi.org/10.1002/jez.b.22526

Grundmann, U., Nerlich, C., Rein, T., & Zettlmeissl, G. (1990). Complete cDNA sequence encoding human β-galactoside α-2,6-sialyltransferase. *Nucleic Acids Research*, *18*(3), 667.

Guarino, F. M., Paulesu, L., Cardone, A., Bellini, L., Ghiara, G., & Angelini, F. (1998). Endocrine activity of the corpus luteum and placenta during pregnancy in Chalcides chalcides (Reptilia, Squamata). *General and Comparative Endocrinology*, *111*(3), 261–270. http://doi.org/10.1006/gcen.1998.7098

He, Y., Hakvoort, T. B. M., Vermeulen, J. L. M., Lamers, W. H., & Van Roon, M. a. (2007). Glutamine synthetase is essential in early mouse embryogenesis. *Developmental Dynamics : An Official Publication of the American Association of Anatomists*, *236*(7), 1865–1875. http://doi.org/10.1002/dvdy.21185

Held-Feindt, J., Hattermann, K., Knerlich-Lukoschus, F., Mehdorn, H. M., & Mentlein, R. (2011). SP100 reduces malignancy of human glioma cells. *International Journal of Oncology*, *38*(4), 1023–1030. http://doi.org/10.3892/ijo.2011.927

Hossain, M. B., Oshima, T., Hirose, S., Wang, J., & Tokumoto, T. (2015). Expression and purification of human membrane progestin receptor α (mPRα). *PLoS ONE*, *10*(9), 1–16. http://doi.org/10.1371/journal.pone.0138739

Itonaga, K., Wapstra, E., & Jones, S. M. (2012). A novel pattern of placental leucine

transfer during mid to late gestation in a highly placentotrophic viviparous lizard. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *318*(4), 308–315. http://doi.org/10.1002/jez.b.22446

Jerez, A., & Ramírez-Pinilla, M. P. (2001). The allantoplacenta of *Mabuya* mabouya (Sauria, Scincidae). *Journal of Morphology*, *146*, 132–146.

Jerez, A., & Ramírez-Pinilla, M. P. (2003). Morphogenesis of extraembryonic membranes and placentation in *Mabuya* mabouya (Squamata, Scincidae). *Journal of Morphology*, *258*(2), 158–178. http://doi.org/10.1002/jmor.10138

Kang, M. J., Fujino, T., Sasano, H., Minekura, H., Yabuki, N., Nagura, H., … Yamamoto, T. T. (1997). A novel arachidonate-preferring acyl-CoA synthetase is present in steroidogenic cells of the rat adrenal, ovary, and testis. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(7), 2880–2884. http://doi.org/10.1073/pnas.94.7.2880

Keverne, E. B. (2014). Mammalian viviparity: a complex niche in the evolution of genomic imprinting. *Heredity*, *113*(2), 138–144. http://doi.org/10.1038/hdy.2014.8

Kirschner, R., Rosenberg, T., Schultz-heienbrok, R., Lenzner, S., Feil, S., Roepman, R., … Berger, W. (1999). RPGR transcription studies in mouse and human tissues reveal a retina-specific isoform that is disrupted in a patient with X-linked retinitis pigmentosa. *Human Molecular Genetics*, *8*(8), 1571–1578.

Kurosawa, N., Kawasaki, M., Hamamoto, T., Nakaoka, T., Lee, Y.-C., Arita, M., & Tsuji, S. (1994). Molecular cloning and expression of chick embryo Gal beta 1, 4GlcNAca2 , 6-sialyltransferase. *European Journal of Biochemistry*, *219*, 375–381.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, *227*, 280–285.

Leal, F., & Ramírez-Pinilla, M. P. (2008). Morphological variation in the allantoplacenta within the genus *Mabuya* (Squamata: Scincidae). *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology*, *291*(9), 1124–1139. http://doi.org/10.1002/ar.20733

Lee, H. J., Jeong, S. K., Na, K., Lee, M. J., Lee, S. H., Lim, J. S., … Paik, Y. K. (2013). Comprehensive genome-wide proteomic analysis of human placental tissue for the chromosome-centric human proteome project. *Journal of Proteome Research*, *12*(6), 2458–2466. http://doi.org/10.1021/pr301040g

Lerch-Gaggl, A., Haque, J., Li, J., Ning, G., Traktman, P., & Duncan, S. A. (2002). Pescadillo is essential for nucleolar assembly, ribosome biogenesis, and mammalian cell proliferation. *Journal of Biological Chemistry*, *277*(47), 45347–45355. http://doi.org/10.1074/jbc.M208338200

Lleras-Forero, L., Tambalo, M., Christophorou, N., Chambers, D., Houart, C., & Streit, A. (2013). Neuropeptides: developmental signals in placode progenitor formation. *Developmental Cell*, *26*(2), 195–203. http://doi.org/10.1016/j.devcel.2013.07.001

Lorenz, L. J., Hall, J. C., & Rosbash, M. (1989). Expression of a Drosophila mRNA is under circadian clock control during pupation, *880*, 869–880.

Maiorana, A., Tu, X., Cheng, G., & Baserga, R. (2004). Role of pescadillo in the transformation and immortalization of mammalian cells. *Oncogene*, *23*(42), 7116–7124. http://doi.org/10.1038/sj.onc.1207916

Martínez-Torres, M., Salcedo-Álvarez, M., Álvarez-Rodríguez, C., Cárdenas-León, M., Luis, J., & Moreno-Fierros, L. (2014). Does the conceptus of the viviparous lizard Barisia imbricata imbricata participates in the regulation of progesterone production and the control of luteolysis? *Animal Reproduction Science*, *148*(3-4), 212–220. http://doi.org/10.1016/j.anireprosci.2014.05.003

Matsson, H., Davey, E. J., Draptchinskaia, N., Hamaguchi, I., Ooka, A., Levéen, P., … Dahl, N. (2004). Targeted disruption of the ribosomal protein S19 gene is lethal prior to implantation. *Molecular and Cellular Biology*, *24*(9), 4032–4037. http://doi.org/10.1128/MCB.24.9.4032-4037.2004

Miralles, A., & Carranza, S. (2010). Systematics and biogeography of the Neotropical genus *Mabuya*, with special emphasis on the Amazonian skink *Mabuya* nigropunctata (Reptilia, Scincidae). *Molecular Phylogenetics and Evolution*, *54*(3), 857–869. http://doi.org/10.1016/j.ympev.2009.10.016

Mohapatra, S. K., Sandhu, A., Singh, K. P., Singla, S. K., Chauhan, M. S., Manik, R., & Palta, P. (2015). Establishment of Trophectoderm Cell Lines from Buffalo (Bubalus bubalis) Embryos of Different Sources and Examination of In Vitro Developmental Competence, Quality, Epigenetic Status and Gene Expression in Cloned Embryos Derived from Them. *PLOS ONE*, *10*(6), e0129235. http://doi.org/10.1371/journal.pone.0129235

Mollereau, C., Simons, M. J., Soularue, P., Liners, F., Vassart, G., Meunier, J. C., & Parmentier, M. (1996). Structure, tissue distribution, and chromosomal localization of the prepronociceptin gene. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(16), 8666–8670. http://doi.org/10.1073/pnas.93.16.8666

Molvarec, A., Tamási, L., Losonczy, G., Madách, K., Prohászka, Z., & Rigó, J. (2010). Circulating heat shock protein 70 (HSPA1A) in normal and pathological pregnancies. *Cell Stress and Chaperones*, *15*(3), 237–247. http://doi.org/10.1007/s12192-009-0146-5

Moremen, K. W., Tiemeyer, M., & Nairn, A. V. (2012). Vertebrate protein glycosylation: diversity, synthesis and function. *Nature Reviews Molecular Cell Biology*, *13*(7), 448–462. http://doi.org/10.1038/nrm3383

Moussatche, P., & Lyons, T. J. (2012). Non-genomic progesterone signalling and its non-canonical receptor. *Biochemical Society Transactions*, *40*(1), 200–204. http://doi.org/10.1042/BST20110638

Murphy, B. F., Belov, K., & Thompson, M. B. (2010). Evolution of viviparity and uterine angiogenesis: Vascular endothelial growth factor (VEGF) in oviparous and viviparous skinks. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, *314 B*(2), 148–156. http://doi.org/10.1002/jez.b.21317

Nanaev, A. K., Kosanke, G., Kemp, B., Frank, H. G., Huppertz, B., & Kaufmann, P. (2000). The human placenta is encircled by a ring of smooth muscle cells. *Placenta*, *21*(1), 122–125. http://doi.org/10.1053/plac.1999.0455

Novakovic, B., Gordon, L., Robinson, W. P., Desoye, G., & Saffery, R. (2013).

Glucose as a fetal nutrient: dynamic regulation of several glucose transporter genes by DNA methylation in the human placenta across gestation. *The Journal of Nutritional Biochemistry*, *24*(1), 282–288. http://doi.org/10.1016/j.jnutbio.2012.06.006

O'Farrel, P. H. (2008). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry*, *22*(5), 629. http://doi.org/10.1016/j.bbi.2008.05.010

Paulesu, L., Cateni, C., Romagnoli, R., Chellini, F., Angelini, F., Guarino, F. M., … Bigliardi, E. (2001). Evidence of Hβ58, a gene involved in mammalian placental development, in the three-toed skink, Chalcides chalcides (Squamata: Scincidae), a viviparous placentotrophic reptile. *Placenta*, *22*(8-9), 735–741. http://doi.org/10.1053/plac.2001.0714

Perkins, D. N., Pappin, D. J. C., Creasy, D. M., & Cottrell, J. S. (1999). Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*, *20*(18), 3551–3567. http://doi.org/10.1002/(SICI)1522-2683(19991201)20:18<3551::AID-ELPS3551>3.0.CO;2-2

Persani, L., Rossetti, R., Di pasquale, E., Cacciatore, C., & Fabre, S. (2014). The fundamental role of bone morphogenetic protein 15 in ovarian function and its involvement in female fertility disorders. *Human Reproduction Update*, *20*(6), 869–883. http://doi.org/10.1093/humupd/dmu036

Price, E. R. (2016). The physiology of lipid storage and use in reptiles. *Biological Reviews*, *9*. http://doi.org/10.1111/brv.12288

Qaidi, S. El, Yang, J., Zhang, J. R., Metzger, D. W., & Bai, G. (2013). The vitamin B6 biosynthesis pathway in streptococcus pneumoniae is controlled by pyridoxal 5'-phosphate and the transcription factor PdxR and has an impact on ear infection. *Journal of Bacteriology*, *195*(10), 2187–2196. http://doi.org/10.1128/JB.00041-13

Rabouille, C., Hui, N., Hunte, F., Kieckbusch, R., Berger, E. G., Warren, G., & Nilsson, T. (1995). Mapping the distribution of golgi enzymes involved in the

construction of complex oligosaccharides. *Journal of Cell Science*, *108*, 1617–1627.

Raijmakers, M. T., Steegers, E. a, & Peters, W. H. (2001). Glutathione S-transferases and thiol concentrations in embryonic and early fetal tissues. *Human Reproduction (Oxford, England)*, *16*(11), 2445–2450. http://doi.org/10.1093/humrep/16.11.2445

Ramírez-Pinilla, M. P., Rueda, E. D., & Stashenko, E. (2011). Transplacental nutrient transfer during gestation in the Andean lizard *Mabuya* sp. (Squamata, Scincidae). *Journal of Comparative Physiology B*, *181*(2), 249–268. http://doi.org/10.1007/s00360-010-0514-6

Renfree, M. B., Suzuki, S., & Kaneko-Ishino, T. (2012). The origin and evolution of genomic imprinting and viviparity in mammals. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *368*(1609), 20120151–20120151. http://doi.org/10.1098/rstb.2012.0151

Robinson, R. S., Woad, K. J., Hammond, A. J., Laird, M., Hunter, M. G., & Mann, G. E. (2009). Angiogenesis and vascular function in the ovary. *Reproduction*, *138*(6), 869–881. http://doi.org/10.1530/REP-09-0283

Rotilio, D., Della Corte, A., D'Imperio, M., Coletta, W., Marcone, S., Silvestri, C., … Donati, M. B. (2012). Proteomics: Bases for protein complexity understanding. *Thrombosis Research*, *129*(3), 257–262. http://doi.org/10.1016/j.thromres.2011.12.035

Rupik, W., Jasik, K., Bembenek, J., & Widłak, W. (2011). The expression patterns of heat shock genes and proteins and their role during vertebrate's development. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *159*(4), 349–366. http://doi.org/10.1016/j.cbpa.2011.04.002

Saben, J., Zhong, Y., McKelvey, S., Dajani, N. K., Andres, A., Badger, T. M., … Shankar, K. (2014). A comprehensive analysis of the human placenta transcriptome. *Placenta*, *35*(2), 125–131. http://doi.org/10.1016/j.placenta.2013.11.007

Scanes, C. G., & Braun, E. (2013). Avian metabolism: Its control and evolution.

Frontiers in Biology, *8*(2), 134–159. http://doi.org/10.1007/s11515-012-1206-2

Schenker, S., Johnson, R. F., Mahuren, J. D., Henderson, G. I., & Coburn, S. P. (1992). Human placental vitamin B6 (pyridoxal) transport: normal characteristics and effects of ethanol. *The American Journal of Physiology*, *262*(6 Pt 2), R966–R974.

Sefton, M., Sánchez, S., & Nieto, M. A. (1998). Conserved and divergent roles for members of the Snail family of transcription factors in the chick and mouse embryo, *3121*, 3111–3121.

Sekijima, M., Tsutsumi, T., Yoshida, T., Harada, T., Tashiro, F., Chen, G., … Ueno, Y. (1999). Enhancement of glutathione S -transferase placental-form positive liver cell foci development by microcystin-LR in aflatoxin B 1 -initiated rats, *20*(1), 161–165.

Self, J. T. (2004). Glutamine synthesis in the developing porcine placenta. *Biology of Reproduction*, *70*(5), 1444–1451. http://doi.org/10.1095/biolreprod.103.025486

Sellix, M. T., & Menaker, M. (2010). Circadian clocks in the ovary. *Trends in Endocrinology and Metabolism*, *21*(10), 628–636. http://doi.org/10.1016/j.tem.2010.06.002

Sites, J. W., Reeder, T. W., & Wiens, J. J. (2011). Phylogenetic insights on evolutionary novelties in lizards and snakes: sex, birth, bodies, niches, and venom. *Annual Review of Ecology, Evolution, and Systematics*, *42*(1), 227–244. http://doi.org/10.1146/annurev-ecolsys-102710-145051

Sparn, H. G., Lieder-Ochs, B. A., & Franke, W. W. (1994). Immunohistochemical identification and characterization of a special type of desmin-producing stromal cells in human placenta and other fetal tissues. *Differentiation*, *56*(3), 191–199. http://doi.org/10.1007/s002580050036

Speake, B. K., Herbert, J. F., & Thompson, M. B. (2004). Evidence for placental transfer of lipids during gestation in the viviparous lizard, Pseudemoia entrecasteauxii. *Comparative Biochemistry and Physiology - A Molecular and Integrative Physiology*, *139*(2), 213–220.

http://doi.org/10.1016/j.cbpb.2004.09.004

Stower, M. J., Diaz, R. E., Fernandez, L. C., Crother, M. W., Crother, B., Marco, A., … Bertocchini, F. (2015). Bi-modal strategy of gastrulation in reptiles. *Developmental Dynamics*, *244*(9), 1144–1157. http://doi.org/10.1002/dvdy.24300

Susnea, I., Bernevic, B., Wicke, M., Ma, L., Liu, S., Schellander, K., & Przybylski, M. (2012). Application of MALDI-TOF-Mass Spectrometry to proteome analysis using stain-free gel electrophoresis. In *TripleC* (Vol. 11, pp. 37–54). http://doi.org/10.1007/128_2012_321

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., … von Mering, C. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Research*, *43*(D1), D447–D452. http://doi.org/10.1093/nar/gku1003

Talbot, N. C., Powell, A. M., Caperna, T. J., & Garrett, W. M. (2010). Proteomic analysis of the major cellular proteins of bovine trophectoderm cell lines derived from IVP, parthenogenetic and nuclear transfer embryos: Reduced expression of annexins I and II in nuclear transfer-derived cell lines. *Animal Reproduction Science*, *120*(1-4), 187–202. http://doi.org/10.1016/j.anireprosci.2010.03.009

Tedeschi, J. N., Kennington, W. J., Tomkins, J. L., Berry, O., Whiting, S., Meekan, M. G., & Mitchell, N. J. (2016). Heritable variation in heat shock gene expression: a potential mechanism for adaptation to thermal stress in embryos of sea turtles. *Proceedings of the Royal Society B: Biological Sciences*, *283*(1822), 20152320. http://doi.org/10.1098/rspb.2015.2320

Thomas, P. (2008). Characteristics of membrane progestin receptor alpha (mPRα) and progesterone membrane receptor component 1 (PGMRC1) and their roles in mediating rapid progestin actions. *Frontiers in Neuroendocrinology*, *29*(2), 292–312. http://doi.org/10.1016/j.yfrne.2008.01.001

Thompson, M. B., & Speake, B. K. (2006). A review of the evolution of viviparity in lizards: Structure, function and physiology of the placenta. *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental*

Physiology, *176*(3), 179–189. http://doi.org/10.1007/s00360-005-0048-5

Tollis, M., Hutchins, E. D., & Kusumi, K. (2014). Reptile genomes open the frontier for comparative analysis of amniote development and regeneration. *The International Journal of Developmental Biology*, *58*(10-11-12), 863–871. http://doi.org/10.1387/ijdb.140316kk

Toyoizumi, R., Shiokawa, K., & Takeuchi, S. (1991). The behavior and cytoskeletal system of chick gastrula mesodermal cells on substrata coated with lines of fibronectin. *Journal of Experimental Zoology*, *260*(3), 345–353.

Tuckey, R. C. (2005). Progesterone synthesis by the human placenta. *Placenta*, *26*(4), 273–281. http://doi.org/10.1016/j.placenta.2004.06.012

Van Dyke, J. U., Beaupre, S. J., & Kreider, D. L. (2012). Snakes allocate amino acids acquired during vitellogenesis to offspring: Are capital and income breeding consequences of variable foraging success? *Biological Journal of the Linnean Society*, *106*(2), 390–404. http://doi.org/10.1111/j.1095-8312.2012.01880.x

Van Dyke, J. U., Brandley, M. C., & Thompson, M. B. (2014). The evolution of viviparity: Molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. *Reproduction*, *147*(1). http://doi.org/10.1530/REP-13-0309

Vermeulen, T., G??rg, B., Vogl, T., Wolf, M., Varga, G., Toutain, A., … H??berle, J. (2008). Glutamine synthetase is essential for proliferation of fetal skin fibroblasts. *Archives of Biochemistry and Biophysics*, *478*(1), 96–102. http://doi.org/10.1016/j.abb.2008.07.009

Wasylyk, C., Schlumberger, S. E., Criqui-Filipe, P., & Wasylyk, B. (2002). Sp100 interacts with ETS-1 and stimulates its transcriptional activity. *Molecular and Cellular Biology*, *22*(8), 2687–2702. http://doi.org/10.1128/MCB.22.8.2687-2702.2002

Wooding, F. B. P., Ramirez-Pinilla, M. P., & Forhead, A. S. (2010). Functional studies of the placenta of the lizard *Mabuya* sp. (Scincidae) using immunocytochemistry. *Placenta*, *31*(8), 675–685. http://doi.org/10.1016/j.placenta.2010.04.001

Zhu, Y., Bond, J., & Thomas, P. (2003). Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(5), 2237–42. http://doi.org/10.1073/pnas.0436133100

Zupan, L. A., Steffens, D. L., Berry, C. A., Landt, M., & Gross, R. W. (1992). Cloning and expression of a human 14-3-3 protein mediating phospholipolysis: Identification of an arachidonoyl-enzyme intermediate during catalysis. *Journal of Biological Chemistry*, *267*(13), 8707–8710.