Gene Expression of Placental Calcium Transporter Proteins During Gestation of *Mabuya* sp

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Trabajo De Grado Para Optar Por El Título De Magister En Biología

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2019

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TITULO: EXPESIÓN DE GENES CODIFICANTES PARA PROTEÍNAS PLACENTALES TRANSPORTADORAS DE CALCIO DURANTE LAGESTACIÓNDE *MABUYA* SP *

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PALABRAS CLAVE: lagartos, viviparidad, placenta, gestación, transporte de calcio, RT-qPCR

DESCRIPCIÓN:

Los lagartos del genero *Mabuya* (Scincidae) son vivíparos y presentan rasgos reproductivos y de biología del desarrollo muy exclusivos, por ejemplo, estos lagartos producen huevos microlecitos y, consecuentemente su patrón de nutrición embrionaria es altamente placentotrófico. Este patrón también se encuentra relacionado con el desarrollo de una de las placentas más complejas y especializadas dentro de reptiles. Esta placenta cumple los procesos metabólicos y fisiológicos requeridos por el embrión durante su desarrollo en la gestación, en estos procesos encontramos el intercambio respiratorio, la transferencia de proteínas, lípidos, glucosa, agua, y algunos iones como el calcio. El calcio es un ion es completamente transportado por la placenta, sin embargo, el posible mecanismo de transporte o las proteínas transportadoras implicadas en su transferencia al embrión siguen sin conocerse. Nuestro objetivo fue detectar y cuantificar por PCR en tiempo real con retrotranscriptasa inversa (RT-qPCR) e inmunofluorescencia la expresión relativa de las proteínas TRPV5/6, Calbindina D28K y D9K, y la bomba ATPasa de calcio (PMCA) en tejidos reproductivos de *Mabuya* sp. Se reportó por primera vez la expresión de TRPV6, Calbindina D28K, D9K y PMCA en estos tejidos. Además, realizamos un énfasis en la expresión de la Calbindina D9K, ya que este trabajó validó su presencia en una especie vivípara no amniota. La expresión relativa reveló un incremento gradual en la expresión de los transcritos durante la gestación, los genes codificantes para transportadores de calcio mostraron un pico en su expresión al final de la gestación, justo cuando están ocurriendo los procesos de mineralización ósea del feto. *Mabuya* sp mostró un mecanismo similar para el transporte materno-fetal de calcio que las especies de mamíferos, sugiriendo que, las proteínas transportadoras de calcio cumplen un rol biológico similar durante la gestación en amniotas.

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* Trabajo de grado

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ABSTRACT

TITLE: GENE EXPRESSION OF PLACENTAL CALCIUM TRANSPORTER PROTEINS DURING GESTATION OF *MABUYA* SP

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KEYWORDS: lizards, viviparity, placenta, gestation, calcium transport, RT-qPCR

DESCRIPTION

Lizards of the genus *Mabuya* (Scincidae) are viviparous with very exclusive reproductive and developmental features, for example, they produce microlecithal eggs and consequently its embryonic nutrition pattern is highly placentotrophic. This pattern is also related with the development of one of the most complex and specialized placentas within reptiles. This placenta fulfills the metabolic and physiological processes required for the embryo development during pregnancy, as the respiratory exchange and the transfer of proteins, lipids, glucose, water, and some ions as calcium. Calcium is completely transport by the placenta, nevertheless, the possible mechanism or transporters implicated in its transfer to the embryo remains unknown. Our aim is to detect and quantify by using RT-qPCR and immunofluorescence the relative expression of TRPV5/6, Calbindin D28K and D9K, and Calcium pump ATPase (all genes encoding calcium transport proteins) in an unnamed species of *Mabuya*. Empty oviducts were taken as control references, while oviducts with oviductal eggs and placental tissues of embryos at early, mid and advanced gestation were the targets. We reported the first assessment of the expression of TRPV6, Calbindin D28K, D9K and PMCA in all the reproductive tissues evaluated in this work in *Mabuya* sp. Moreover, we emphasize the Calbindin D9K expression in *Mabuya* sp, and validate its presence in a non-mammalian viviparous species. Relative expression reveals a gradual increase in the calcium transcripts through pregnancy; calcium transporter genes showed their expression peak at the end of gestation when bone mineralization of the fetus occurs. *Mabuya* sp shows a similar mechanism for maternal-embryo calcium transport as mammalian species; suggesting that, calcium transport proteins play a similar biological role during pregnancy within amniotes.

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* Master Thesis

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Introduction

Squamate reptiles (lizards and snakes) are one of the most diverse groups within amniotes. They exhibit an evolutionary transition in their parity mode from oviparity (egg-laying) to viviparity (live-bearing); viviparity has evolved independently more than 150 times in vertebrate lineages, 115 from those evolved in squamates (Blackburn, 2015). Squamates are also known for the wide diversity degree of embryonic nutrition patterns. These patterns go from lecithotrophy (derived from reserves accumulated in the egg yolk, oviparous and most viviparous lineages) to placentotrophy (a type of conspicuous matrotrophy where the mother supplies substantial nutrition to the embryo through the placenta, viviparous lineages). Highly placentotrophic squamates are rare, as it has only been documented in four to five lizard lineages belonging to Scincidae family (Blackburn & Flemming, 2009).

Remarkably, within these lineages of Scincidae, neotropical lizards of the genus *Mabuya* shows reproductive and developmental traits associated to one of the highest degrees of placentotrophy known among squamates. Its species produce microlecithal eggs with a minimum amount of yolk (Gómez & Ramírez-Pinilla, 2004; Hernández-Franyutti, Uribe & Guillette, 2005; Vieira, de Perez & Ramírez-Pinilla, 2010), and therefore the intraoviductal development (that takes 9 to 10 months) depends completely on placental nutrient transfer (Vitt & Blackburn, 1983; Ramírez-Pinilla, 2006). Consequently, the placenta of *Mabuya* is also one of the most complex in reptiles (Leal & Ramírez-Pinilla, 2008; Blackburn & Flemming, 2009), and has very unusual specializations implicate to the nutrient transfer during the embryo development (Jerez & Ramírez-Pinilla, 2001, 2003; Vieira *et al.*, 2007). All these features make them particularly important as they contribute with a system that allows performing comparative physiological, metabolic, and developmental biology investigations.

Previous studies on Colombian *Mabuya* have provided clues about the placental morphology and its physiology. Similar to eutherian mammals, the placenta fulfills the fundamental functions for the absorption and transfer of nutrients such as water, glucose, lipids (cholesterol, vitamin E and fatty acids), proteins (related to metabolism, energy, signaling, embryonic development, progesterone synthesis and its receptors), and ions of potassium, sodium, magnesium, and calcium (Ramírez-Pinilla, 2006; Wooding, Ramírez-Pinilla & Forhead, 2010; Ramírez-Pinilla, Rueda & Stashenko, 2011; Hernández-Díaz, Torres & Ramírez-Pinilla, 2017; Duarte-Méndez, Quintero-Silva & Ramírez-Pinilla, 2018).

Calcium is one of the most essential ions required during gestation, not only for bone mineralization, but also is crucial for pregnancy establishment, metabolic processes, muscle contraction, embryo growth and fetal homeostasis (Reviewed in Belkacemi *et al.*, 2005; Tivane *et al.*, 2013). Understanding the regulatory and physiological mechanisms for calcium transport in maternal and embryonic tissues, provide clues of what physiological processes were involved during the transition from oviparity to viviparity in reptiles (Stewart & Ecay, 2010), as well as knowledge related to its convergent evolution with mammals (Brandley *et al.*, 2012; Van Dyke, Brandley & Thompson, 2014).

Calcium placental transport in *Mabuya* exhibits its highest index value during pre-parturition stage, that is when the placenta is mature and fetus skeletal mineralization occurs (Ramírez-Pinilla, 2006; Ramírez-Pinilla *et al.*, 2011). Likewise, immunohistochemical analysis for Calcium-binding proteins Calbindin-D9K and calbindin-D28K showed multiple routes for calcium transport in the placenta (Wooding *et al.*, 2010). However, it remains unknown if the antibodies used in previous studies, which are specific for mammalian calcium binding proteins, cross-react with non-mammalian homologous proteins. Consequently, the detection of these specific proteins in

Mabuya placental tissues must be further be analyze with tools that allow the specific detection of calcium transporting proteins or their coding transcripts.

Hence, our aim in this study is to quantify the relative expression of mRNA encoding calcium transporters in tissues of different gestation stages in *Mabuya* sp, by using Reverse Transcription quantitative Polymerase chain reaction PCR (RT-qPCR). Taking into account, the newly availability of the placental transcriptome of *Mabuya* (Cornelis *et al.*, 2017) give us the chance to evaluate multiple genes profiles in this valuable non-model organism. We also want to locate the most important proteins expressed in advanced placental tissues using immunofluorescence, compare its expression to previous reports, and with the physiological processes given during pregnancy in other amniotes.

1. Materials and methods

1.1 Studied Population

Mabuya sp IV (as is not yet named) is a candidate specie due to its notable genetic divergence. *Mabuya* sp IV is widely distributed among mid elevation sites (from 65 to 1550 m) across the Colombian andes. Females (n=13) were captured in the municipalities previously sampled by Pinto-Sánchez *et al*. (2015) in the department of Santander, Colombia. The animals used in this study were collected under the license given to the Universidad Industrial de Santander (Permiso Marco de recolección de especímenes silvestres de la diversidad biológica con fines de investigación científica no comercial, Resolución 004, January 22, 2014 from Autoridad Nacional Ambiental). Specimens correspond to the following registers of the Colección Herpetológica, Museo de Historia Natural of the University: UIS-MNH-R:3893, 3895-3897, 3902, 3912-3914, 3920, 3922, 3926-3928.

All work conducted with the animals was consistent with government guidelines on the ethical treatment of animals and all applicable regulations, and follows the considerations of The Herpetological Animal Care and Use Committee (HACC) (American Society of Ichthyologists and Herpetologists) (Beaupre *et al.*, 2004).

1.2 Tissue sampling and processing

Individuals were kept over a week under feeding and temperature controlled. Pregnant females were euthanized by intrathoracic injection of lidocaine 2% v/v. In total four kidneys, five oviducts, seven oviducts with oviductal eggs, ten embryonic chambers in early (blastula, gastrula or neurula) gestation, eight placental tissues at mid gestation (limb bud stage); and eight placental tissues at late gestation were dissected out. Followed, tissues were immersed in RNAlater (Qiagen) or buffered paraformaldehyde 4% v/v (PFA), and dehydrated in a gradual series of ethanol.

Embryos at mid and late gestation were dissected from the embryonic chambers and only placental tissues (the conjunction between maternal uterus and extraembryonic membranes) were fixed in RNA later or PFA 4%. The embryos were used to classify the embryonic developmental stages under stereo microscope according to the Dufaure & Hubert (1961) table.

1.3 Primer Design

We isolated transcripts of interest from a previously transcriptome assemblage of the *Mabuya* placenta (Cornelis *et al.*, 2017) using stand-alone blast (Altschul *et al.*, 1990). We were interested in exploring the possible route for calcium transport and absorption that has been reported on mammals (Nijenhuis, Hoenderop & Bindels, 2005). Specific primers were designed according to the guide for design real time PCR primers proposed by Thornton & Basu, (2011) in Primer3 and IDT software. Actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were tested as

referenced genes and five calcium transport proteins were evaluated: Calbindin-D9K (CaBP-9k), Calbindin-D28K (CaBP-28K), Transient Receptor Potential Cation channel subfamily V member 5 (TRPV5), Transient Receptor Potential Cation channel subfamily V member 6 (TRPV6), and the Plasma Membrane Calcium ATPase (PMCA). Gene description and primer sequences are provide in Table 1.

1.4 RNA isolation

Total RNA was extracted with InviTrap® Spin Universal RNA Mini Kit according to manufacturer's recommendations. RNA concentration was measured by spectrophotometry using a NanoDrop® ND-1000, while the purity and integrity were assessed with the optical density ratio at 260/280 (≥ 1.8) and by denaturing gel electrophoresis analysis. Samples were mixed in equal volumes with 2X RNA loading dye (NEB®) and heated at 65 °C for 10 min. Gel was prepared by melting the agarose in DEPC water, and by adding 10X MOPS running buffer (0.4 M Mops pH 7.0, 0.1 M sodium acetate, 0.01 M EDTA) and formaldehyde. Finally, the gel was run in 1X MOPS running buffer. The bands obtained were compared with the ssRNA ladder from New England BioLabs® (NEB).

1.5 cDNA synthesis and RT-qPCR

We used 1 μg of RNA per sample to reverse transcribe with ProtoScript II® First Strand cDNA. The RT primer Mix contained both oligo dT and random primers to obtain a maximum number of cDNA transcripts. Real time PCR was performed with Luna Universal qPCR Master Mix (NEB) in a Mastercycler® ep realplex 4 (Eppendorf) under the following conditions: at 95°C for 2 min; followed by 40 cycles of at 95°C for 15 s, primer-dependent temperature annealing for 15 s, and 72°C for 20 s.

The lack of primer dimers was checked with a melting curve analysis, and by agarose gel electrophoresis 2% (w/v). In order to avoid contamination, a no-template control was included. The reaction efficiency of each gene was calculated from a 5 serial cDNA dilution standard curve, and relative gene expression was calculated using the $2^{-\Delta\Delta C}$ method (Livak & Schmittgen, 2001).

Oviducts of non-pregnant females were the control samples, whereas oviducts with oviductal eggs, placentas from early, mid and advanced pregnant females were the target samples. Biological triplicates were examined for RT-qPCR analysis, and four technical replicates were analyzed for each biological sample. Kidney tissues were used as positive controls as this organ is one of the main sites for calcium active transport and (re)absorption, also because the CaBP-28K, CaBP-9K, PMCA, and TRPV6 are the strongly expressed.

1.6 Data analysis

Efficiency percentage for each gene was calculated with two samples of kidneys and late placental tissues. The relative expression by the $2^{-\Delta\Delta C}$ method was evaluated according to the single gene efficiency adjustment. All measurement data were expressed as mean ± standard deviation (SD) and Kruskal Wallis statistical analyses were performed since data did not fit the assumptions of a parametrical test. Statistical software Xlstat v.8.0. was used and a P value of < 0.05 was considered statistically significant.

1.7 Immunofluorescence analyses

Placental fixed tissues at advanced gestation were paraffin-embedded, sectioned at 5 µm thick sections in a rotatory microtome (Leica Biosystems®), and placed onto silane-treated coated slides. Sections were then deparaffinized and rehydrated consecutively in a gradient of ethanol to water.

Tissue antigens were retrieved using 10 mM sodium citrate buffer pH 6.0, 0.05% tween-20 for 20 min at 98°C and cooled down in ice water for 10 min. Unspecific binding was blocked with 10% in blocking serum (1:1000 dilution, Santa Cruz Biotechnology®, SCBT) in 1X PBS with 0.4% Triton X-100 for 1 hour at room temperature. Subsequently, sections were washed three times in PBS and primary antibodies for CaBP-28K (1:100, SCBT®) and CaBP-9K (1:100, Anti-S100G antibody, Abcam®) in blocking buffer were added overnight at 4°C. Staining was visualized with fluorochrome-conjugated secondary antibodies Anti-mouse Alexa Fluor 488® (SCBT®) or Anti-rabbit IgG (H+L), F(ab')2 Fragment Alexa Fluor® 594 (Cell Signaling Technology®) correspondingly. Nuclei were stained using 1 µg DAPI. Negative controls were performed by not adding the primary antibodies. Slides were also dyed with hematoxylin and eosin stain. Slides were watched in an Axio Scan Z1 microscope (ZEISS).

2. Results

Our results confirm the presence of CaBP-9K, CaBP-28K, PMCA and TRPV6 mRNA in non-pregnant oviducts, oviducts with oviductal eggs, embryonic chambers at early developmental stages, and placental tissues at early, mid, and advanced gestation in the highly placentotrophic lizard *Mabuya* sp. We also evaluated the mRNA expression of β-actin and GADPH as reference genes. The expression of these two candidate genes was assayed among all the tissues. β-actin was chosen as the reference gene because its cycle quantification value (Cq) was stable during the gestation.

Melting curve analyses of each gene have a single peak and primer dimmers, or other nonspecific amplification products were not detected. Moreover, the agarose gel electrophoresis shows the amplification of the four genes with a single band. The size of the band matches the one

of the amplicon size indicating the primers specificity. Nevertheless, although we test two different primer sets for TRPV5, we did not find a positive amplification.

Comparisons of transcripts abundance reveal a consistent dominance expression of CaBP-28K in all the tissues. Interestingly, there was a gradual increase in the expression of the four genes during gestation, and the overall transcripts level of CaBP-9K, CaBP-28K, PMCA and TRPV6 was highest in placental tissues from mid to late gestation (Fig. 1). Fold changes obtained by real time PCR indicate a downregulation of calbindin D28K and calbindin D9K in early gestation compared to oviducts with oviductal eggs. Whereas TRPV6 has a higher fold change in early gestation compared to the other transcripts ($P=0.031$). CaBP-28K and PMCA fold change was greatest in mid and advanced placental tissues ($P<0.05$) (Fig. 1).

Currently, reptile antibodies for immunofluorescence that react positively to calcium transporter proteins are not available. Hence, we try out monoclonal mammalian CaBP-28K, since it is the most significantly expressed transcript through gestation. CaBP-9K was evaluated due to its particularly limited expression within amniotes, especially in view of *Mabuya* sp is the only viviparous squamate in which expression have been found until now.

CaBP-28K intensely stains the luminal uterine and the chorionic epithelia (specifically in the giant binucleated cells) in the placentome and paraplacentome on the embryonic hemisphere of the mature placenta. There is no expression in the uterine glands (in the entire tissue) or in the uterine syncytium of the placentomes (Fig. 2 and 3). Otherwise, CaBP-9K protein is located in the apical border and microvillar surfaces of the giant cells in the chorionic epithelium of the placentome and paraplacentome. Furthermore, the strongest label is in the cytoplasm of the uterine epithelium in the paraplacentome, with negative detection in the uterine glands. Moreover, CaBP-

9K is detect in the basal membrane of the uterine epithelium in the entire abembryonic hemisphere of the embryonic chamber, including the absorptive plaques (Fig. 4, 5 and 6).

3. Discussion

3.1 RT-qPCR - Gene expression

We present the first approach of direct and specific relative quantitative mRNA measurement for calcium transporters (CaBP-28K, CaBP9K, PMCA and TRPV6) in any viviparous placentotrophic squamate lizards. Our work highlights the expression of CaBP-9K in the placenta of a non-mammalian amniote species (Wooding *et al.*, 2010), in this case in the specialized placenta of *Mabuya* sp through all gestation. Here, we also have taken advantage of the valuable newly generated data set of high-throughput RNA sequencing of the *Mabuya*'s placenta transcriptome (Cornelis *et al.*, 2017) for the evaluation of two reference genes, five target genes encoding calcium transporters and by the way a general RNA-seq outcome validation.

Calcium dynamics during pregnancy in *Mabuya* sp follows a similar pattern to other viviparous squamates and amniotes. The expression of CaBP-28K, CaBP-9K, PMCA and TRPV6 in *Mabuya* reproductive tissues suggest a role in the establishment and maintenance of pregnancy. Our results also show that maternal–fetal calcium transport is crucial for fetal Ca^{2+} homeostasis and bone mineralization during the last developmental stages. Although Ca^{2+} is delivered to embryos early in gestation in *Mabuya* sp, its major secretion phase is shifted to mid and late developmental stages, when the uterine and extraembryonic tissues are specialized, and the embryos have the machinery to incorporate calcium to supply their growth demands (Ramirez-Pinilla *et al.*, 2006; Ramírez-Pinilla *et al.*, 2011).

Regarding the underlying Ca^{2+} absorption and transport mechanisms, breakthrough discoveries on mammalian kidney, intestine, and bone have vastly expanded our knowledge. Calcium transport can be given mainly by two pathways, the first is along the paracellular pathway by passive diffusion through the narrow junctions of the epithelium or between adjacent cells (Todd, Juraj & Henrik, 2014). On the other hand, Ca^{2+} is actively transported in the transcellular route, the ions enter the cell via the selective apical plasma membrane channels TRPV5 and TRPV6, then bind to cytosolic proteins CaBP-9K and 28K, and finally are extruded across the basolateral plasma membrane by PMCA or the sodium-calcium exchanger protein NCX. Based on this model, we evaluated the encoding transcripts mentioned during *Mabuya* sp gestation, since they contribute as markers to evaluate Ca^{2+} transport in several tissues (Belkacemi *et al.*, 2005; Cordeiro & Hincke, 2016), including uterus and placenta of other squamate reptiles (Table 2).

The epithelial calcium channels TRPV5 and TRPV6 have been extensively studied in tissues controlling Ca^{2+} homeostasis. TRPV5/6 are highly selective channels of Ca^{2+} in the TRPV superfamily and play an important role in Ca^{2+} (re)absorption by the mammalian kidney and intestine. Both proteins are co-expressed in some tissues, but, in contrast to TRPV5, TRPV6 is more ubiquitously expressed e.g., intestine and kidney of laying hens (Taylor *et al.*, 2011), esophagus, stomach, small intestine, colon, kidney, placenta, pancreas, prostate, uterus, salivary gland, and sweat gland of mammals (Nijenhuis *et al.*, 2005; Lee *et al.*, 2009). Whereas TRPV5 is relatively restricted to mammalian kidney, mice placenta and human syncytiotrophoblast (Haché *et al.*, 2011; Yang, Ahn & Jeung, 2015).

TRPV6 has not been reported yet in reproductive tissues as oviducts or placental tissues from any viviparous squamate. TRPV6 mRNA expression was the second more expressed in placental tissues at the end of gestation in *Mabuya* sp (Fig. 1). Our results show a similar pattern as in mice,

human and bovine uterine endometrium and placentomes, in which the highest TRPV6 and CaBP-9K level expression was found in the last trimester of pregnancy, thus these proteins have a functional role in Ca^{2+} metabolism in gestation (Lee *et al.*, 2009; Sprekeler, Kowalewski & Boos, 2012).

Calbindin proteins not only bind and transport calcium but also protect the cells from high concentrations of Ca^{2+}, by buffering excessive intracellular levels of free ions (Belkacemi *et al.*, 2003). CaBP-9K was first described in *Mabuy*a sp mature placenta by Wooding *et al*. (2010), and it has been an exclusive report on the placenta of a non-mammalian species. In this study, we corroborate its expression, but interesting we discover that CaBP-9K not only is expressed in placental tissues but also in non-pregnant oviducts, and in oviducts with oviductal eggs and with incubatory chambers during the entire gestation.

Calbindin-D9K has not been reported in any squamate species up to now. CaBP-9K is found in mammalian tissues as intestine, uterine epithelium, and placenta (in both maternal and fetal epithelia), and is involved on mammalian maternal-fetal calcium transport (Wooding *et al.*, 2000, 2015); however, it is not expressed in the uterine or extraembryonic membranes of chicken tissues (Zanello, Boland & Norman, 1995).

Our results show high expression CaBP-9K and CaBP-28K in *Mabuya* sp oviducts with oviductal eggs, and then the down regulation during the early pregnancy (Fig. 1), suggesting the involvement of these proteins in the embryo establishment in the oviduct. This pattern is seeing during mouse embryo implantation, where the endometrial calbindin D28K and 9K are critical for this process. Similar to *Mabuya* sp, transcript expression of encoding the calcium binding proteins D28K and D9K increased just before mouse uterine implantation, and after the attachment the

expression level goes down (Tatsumi *et al.*, 1999). CaBP-28K and CaBP-9K help to the implantation by protecting the embryo from any hypercalcemic effect, and regulate the calcium concentration in the uterine cavity (Luu *et al.*, 2004).

Most studies in oviparous and viviparous lizards and snakes have focused on the detection of intracellular CaBP-28K and PMCA through gestation. CaBP-28K seems to be the most important calcium transporter during gestation of *Mabuya* as it has the highest fold change (Fig. 1). CaBP-28K relevance is also reflected in a wide variety of tissues including the uterine and chorionic epithelia of other squamate reptiles (Table 2), the kidney, liver, heart, egg shell gland, yolk sac, uterus and chorioallantoic membrane of chicks (Sechman *et al.*, 1994; Brionne *et al.*, 2014). As well as in the human uterus, trophoblast cells, cerebellum, pancreas and endometrium, where regulates endometrial receptivity (Yang *et al.*, 2011).

Otherwise, calcium APTase pumps are critical in most cells, as they regulate general calcium signaling. This study reports the first assessment of PMCA expression in several reproductive tissues of *Mabuya*. PMCA plays an important role in extrusion of cytosolic Ca^{2+} from the cell (Belkacemi *et al.*, 2005; Yang *et al.*, 2013), and it is the major mechanism for deposition of the calcareous eggshell in oviparous reptiles as in the lizard *Lampropholis guichenoti* (Thompson *et al.*, 2007) and in chicks (Akins & Tuan, 1993). The presence of high levels of Ca^{2+} ATPase pumps in mid-late pregnancy in placental tissues of *Mabuya* sp, in the uterine and glandular epithelial cells (shell glands) of *Pseudemoia spenceri* and *P. entrecasteauxii*, implies that pumps are involved in calcium transport out of the epithelial cells and into the uterine lumen, where is received by the extraembryonary membranes of the developing embryos in species with complex placentas (Herbert, Murphy & Thompson, 2006).

3.2 CaBP-28K and CaBP-9K Immunofluoresence

Mabuya has the highest placental complexity known so far among Reptilia. This placenta converges in many morphological and physiological aspects with those in eutherian mammals, but it also shows very remarkable features (Ramirez-Pinilla *et al.*, 2006). These features are chiefly related to the very early yolk sac placenta regression and the development of an allantoplacenta that is specialized in nutrient transfer.

The regionalization and specializations of the mature placenta located in the embryonic hemisphere include the placentome, paraplacentome, and the chorionic areolas, all associated with nutrient transfer. On the abembryonic hemisphere (yolk sac is on the embryonic ventral side) are the absorptive plaques, and the respiratory segments (Jerez & Ramírez-Pinilla, 2001, 2003; Leal & Ramírez-Pinilla, 2008).

Immunochemical location of CaBP-28K and CaBP-9K in the mature placenta of *Mabuya* sp bring comparable locations between the chicken and mammalian polyclonal antibodies respectively tested by Wooding *et al*. (2010), and the mouse monoclonal and rabbit polyclonal antibodies examined in this study.

Antibodies in both studies strongly labelled CaBP-28K in the cytoplasm of the giant binucleate cells in the chorionic epithelium of the placentome (Fig. 3) and paraplacentome. No expression was found in the areolar structures, respiratory segments, yolk sac or in the uterine glands. Nevertheless, we did find a positive tag on the uterine epithelium on the entire chamber (Fig. 2). Similar detection is reported for CaBP-9K. CaBP-9K is located on the microvillar apical borders of the giant cells in the chorionic epithelium of the placentome and paraplacentome, and absorptive plaques, and in the basal plasmalemma of the uterine syncytium in the placentome (Wooding *et al.*, 2010) and of the uterine epithelium of the absorptive plaques.

The expression of CaBP-28K in uterine epithelium and embryonic membranes of oviparous and viviparous squamates (Table 2), and the expression of CaBP-9K in specific regions of placental tissues in *Mabuya* sp, reflect specific functional cell specializations for calcium transport. For example, both calcium binding proteins in placentome and paraplacentome ideally fit this role, due to the interdigitation of the uterine tissue, that greatly increases the surface area for placental exchange (Ramírez-Pinilla *et al.*, 2006; Vieira *et al.*, 2007; Leal & Ramírez-Pinilla, 2008).

Regardless of its origin (yolk, eggshell, or placenta), calcium must cross the uterine epithelium and the chorionicallantoic ectoderm, or yolk sac endoderm before entering the embryonic circulation and becoming available to support growth and development (Fregoso, Stewart & Ecay, 2012). Therefore calcium uptake from the uterine epithelium/eggshell is absorbed from uterine epithelium by chorionic epithelial cells and lastly transported to underlying embryonic capillaries (Stewart *et al.*, 2009).

4. Conclusions

Calcium transporters evaluated in this study seem to be conserved and play similar biological roles in reproductive tissues within amniotes and in placentotrophic species, maybe due to convergent evolution and tissue homologies. We also provide the first evidence that CaBP-28K, CaBP-9K, PMCA and TRPV6 are involved in the establishment, maintenance of gestation and maternal–fetal Ca^{2+} transport in the viviparous placentotrophic specie *Mabuya* sp. Our results also emphasize *Mabuya* sp as the only reptile species where the presence of CaBP-9K is reported in oviducts, oviductal eggs, embryonic chambers and placental tissues nowadays. Therefore, the expression of CaBP-9K might be restricted to species with complex placenta and high degree of placentotrophy, as *Mabuya* and mammals. New questions arise from the development of this work:

¿why is calbindin D9K mainly associated with calcium metabolism and transport in reproductive tissues?, ¿are there any placental nutrient transporters that evolve *de novo* in placentotrophic species?, ¿how is the mechanism for the transport of all the other ions, lipids and proteins during gestation in *Mabuya* sp?.

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Tables

Table 1. Primers used in qPCR experiments. Primers are listed 5' → 3'

Gene	Forward primer	Reverse primer	Tm [a]	Efficiency [b]
B-Actin	TCCTCCCTGGAGAAGAGTTATG	CCAAGAAAGAAGGCTGGAAGAG	62.1	94
GADPH	TTGAAGTCACAGGAGACAACC	GGAGAAACCAGCCAAGTATGA	59.9	99
CaBP-9K	CAGGCACAGTGCTGTCTAAAG	GCCAGCTATCCCAAAGTGAAT	60.9	93
CaBP-28K	GAGCGAGCTCTACAATCCCTAT	TCAGCAGGCACGAAAGAATG	60	88
TRPV5	GATTTGTCGGGCCTCTCTTT	GCCTGTAACCTCTTTCCTCTAC	-	-
TRPV5 - 1	TGTGAGGTCGTAGAGGAAAGA	TCAAACTGGCTGGAGTTGAG	-	-
TRPV6	CGCCCAGTAACACATGGTATAG	CTGTGTGTTGCCCATCTAAGT	62.1	99
PMCA	CCAAGGGACCGTGATGATATTG	CCTGCTGGAAAGTCTCTGAATG	62.1	99

[a] Annealing temperature

[b] PCR Efficiency expressed in percentage

Table 1. Calcium transporters expression and evaluation on maternal and extraembryonic membranes of squamate reptiles. Data is organized according to the complexity of the embryonic nutrient transfer.

Species	***Parity/ Nutrition***	***Protein***	***Method***	***Labelling***		***References***
				Uterus	**Embryonic**	
Lampropholis guichenoti	Oviparous Lecithotrophic	PMCA	IF	Glands and epithelium	-	(Thompson *et al.*, 2007)
Virginia striatula	Viviparous Lecithotrophic	CaBP-28K, PMCA	WB	-	Yolk sac splanchnopleure and chorioallantois	(Fregoso *et al.*, 2012)
Elaphe guttata	Viviparous Lecithotrophic	CaBP-28K	WB	-	Yolk splanchnopleure and chorioallantoic membrane	(Ecay *et al.*, 2004)
Zootoca vivipara	Viviparous Lecithotrophic	CaBP-28K	WB	-	Chorioallantoic membrane	(Ecay *et al.*, 2017)
Niveoscincus metallicus; N. ocellatus	Viviparous Placentotrophic	PMCA	IF	Glandular epithelium	-	(Herbert *et al.*, 2006)
Pseudemoia pagenstecheri	Viviparous Placentotrophic	CaBP-28K, PMCA	IHC	Shell glands and uterine epithelium	Chorioallantoic and omphaloplacenta	(Stinnett, 2011)
P.spenceri; P. entrecasteauxii	Viviparous Placentotrophic	PMCA	IF	Shell glands and uterine epithelium	Chorioallantoic and omphaloplacenta	(Herbert *et al.*, 2010)
Mabuya sp	Viviparous Placentotrophic	CaBP-28K	IHC	-	Giant binucleate cells cytoplasm in the chorionic epithelium, and some absorptive plaques	(Wooding *et al.*, 2010)
Mabuya sp	Viviparous Placentotrophic	CaBP-9K	IHC	-	Placentomal, paraplacentomal and plaque microvillar borders	(Wooding *et al.*, 2010)

					probably on the chorionic epithelium, and the placentome uterine epithelial syncytium	
Mabuya sp	Viviparous Placentotrophic	CaBP-28K	IF	Uterine epithelium, with no stain in the glands	Cytoplasm of the giant binucleate cells in the chorionic epithelium of the placentome and paraplacentome	Present study
Mabuya sp	Viviparous Placentotrophic	CaBP-9K	IF	Uterine epithelium, with no stain in the glands	Apical cytoplasm of the giant binucleate cells in the chorionic epithelium of the placentome, chorionic cells of the paraplacentome, and absorptive plaques	Present study
Mabuya sp	Viviparous Placentotrophic	CaBP-28K, CaBP-9K, PMCA, TRPV6	RT-qPCR	Oviducts	Oviducts with oviductal eggs, placental tissues at early, mid and late gestation	Present study

IHC: Immunohistochemistry; IF: Immunofluorescence; WB: Western blotting; RT-qPCR: Reverse transcription real time PCR

Figures

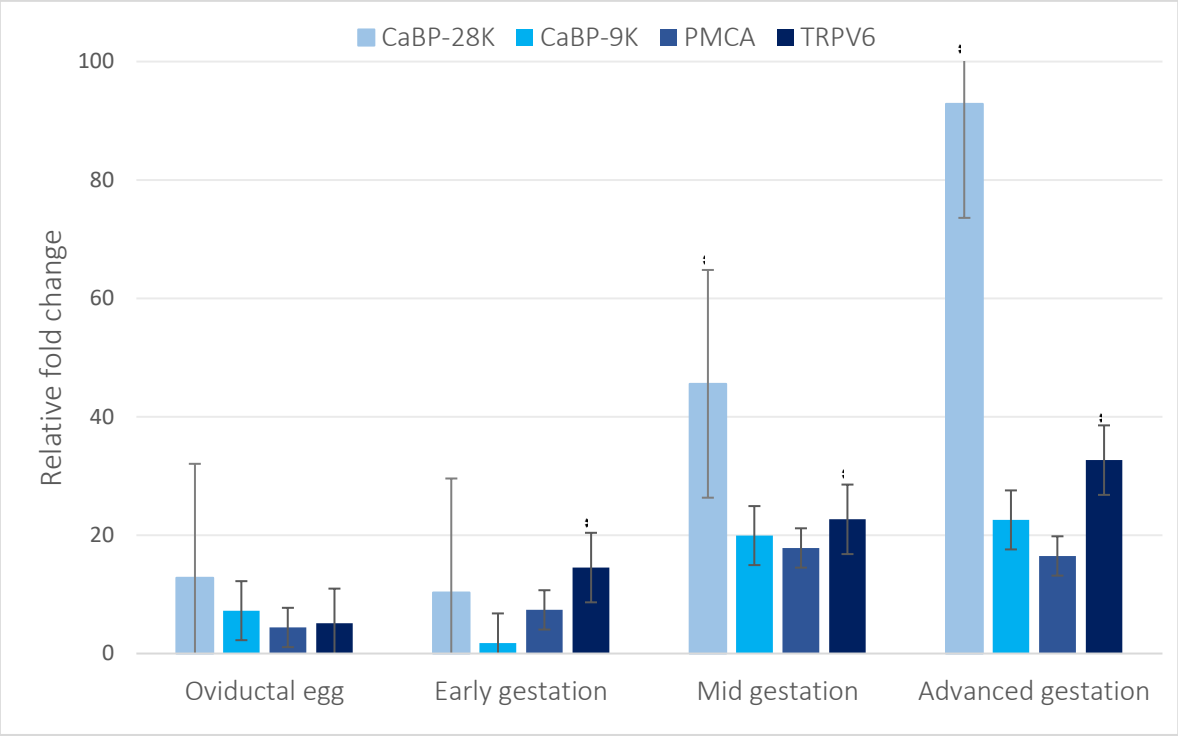

Figure 1 Fold-change comparison in the placenta through pregnancy relative to gene expression in the oviducts of non-pregnant females ($2^{-\Delta\Delta C}$ relative method). A gradual increase expression is evidenced for CaBP28K, CaBP-9K, PMCA and TRPV6 genes through pregnancy. CaBP-28Ktranscripts show the highest upregulation in mid and late gestation. Asterisk indicate significant differences, $P<0.05$.

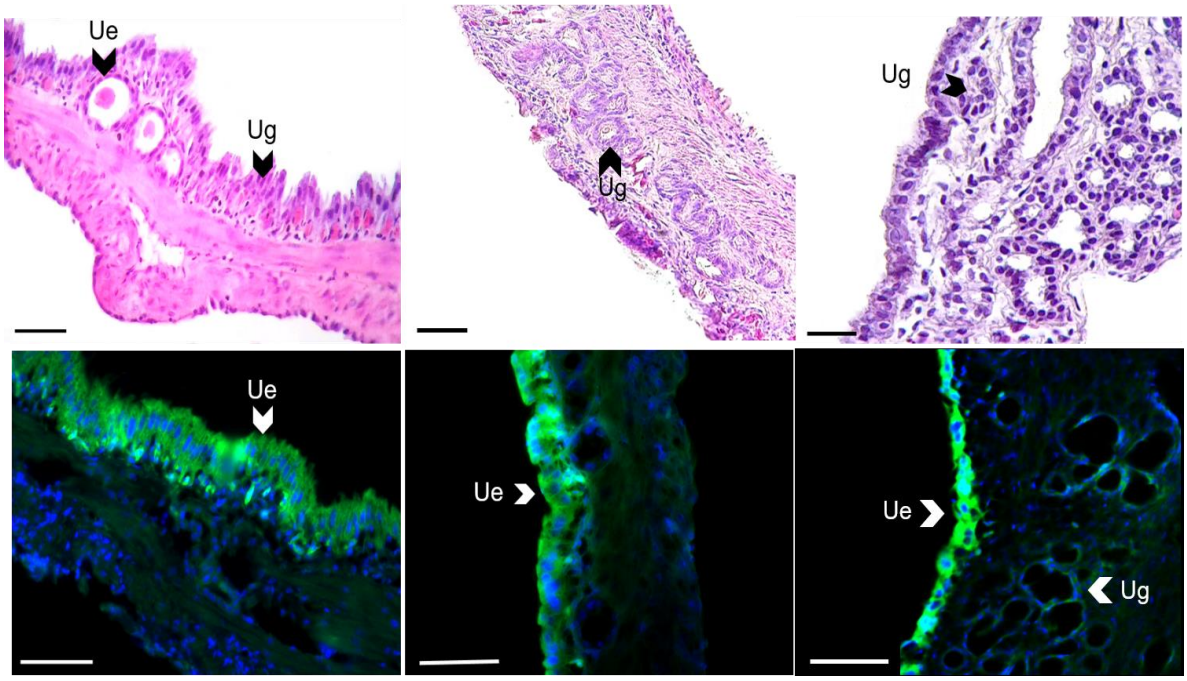

Figure 2 Histological sections of *Mabuya* sp placental tissues at late development. Calbindin-D28K immunolabelling is positive in the uterine epithelium at the embryonic hemisphere, in contrast the uterine glands do not stain. Upper images represent hematoxylin and eosin staining; lower images show the immunofluorescence labelling. Ug. Uterine gland; Ue. Utherine epithelium. Nucleus (blue) are stain with DAPI. Scale bar: 30µM.

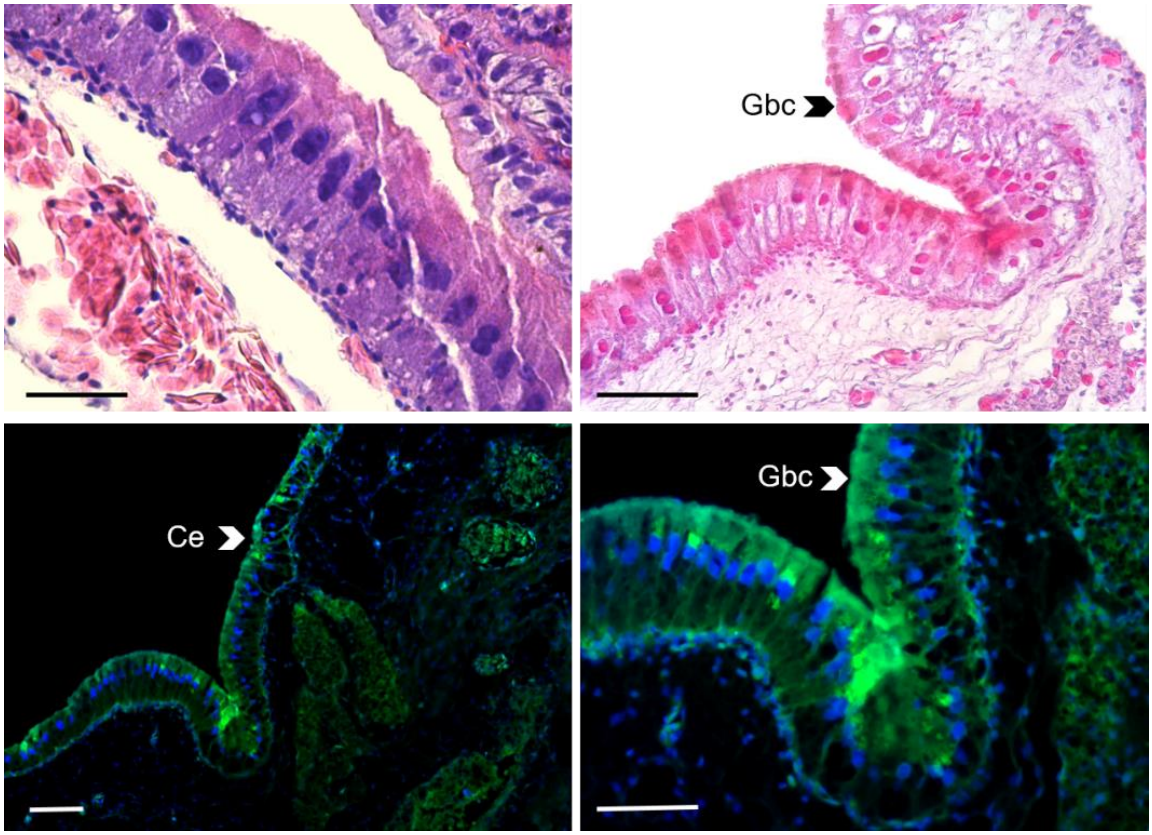

Figure 3 Histological sections of *Mabuya* sp placental tissues at late development. In the paraplacentome CaBP-28K labels the giant cells in the chorionic epithelium. Upper images represent hematoxylin and eosin staining; lower images show CaBP-28K immunolabelling. Ce, chorionic epithelium; Gbc, giant binucleated cells. Nucleus (blue) are stain with DAPI. Scale bar: 30µM.

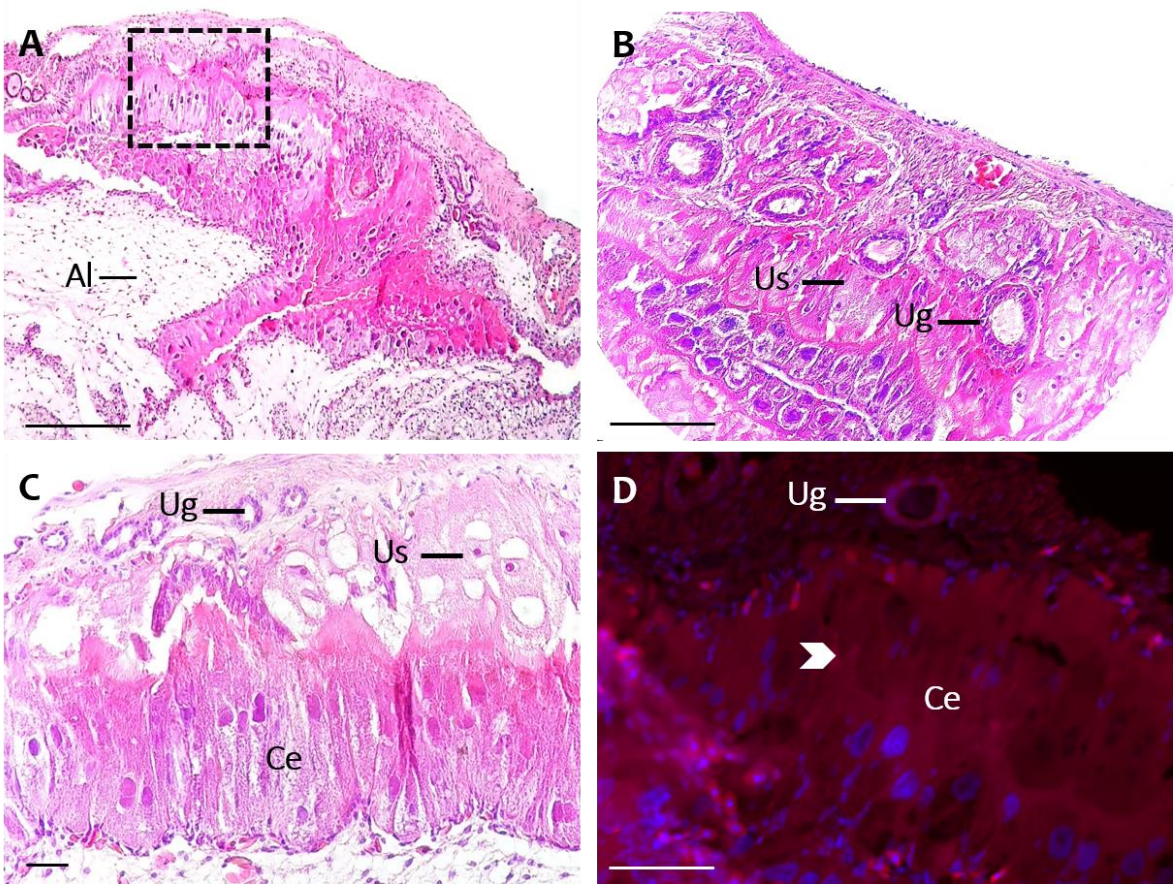

Figure 4 Histological sections of *Mabuya* sp placental tissues at late development. A. Paraplacentome section of *Mabuya* sp in the embryonic hemisphere. B. Placentome section showing the strong interdigitation between the chorionic epithelium and the uterine syncytium. C. Inset of the chorionic cells of the placentome. D. CaBP-9K labeling the apical border of the giant cells in the chorionic epithelium of the placentome. A, B and C images represent hematoxylin and eosin staining; D image shows CaBP-9K immunolabelling in the chorionic epithelium of the placentome. Al, allantois; Ce, chorionic epithelium; Us, uterine syncytium; Ug, uterine glands. Nuclei (blue) are dye with DAPI. Arrows indicate the strong positive labeling. Scale bar: glands.

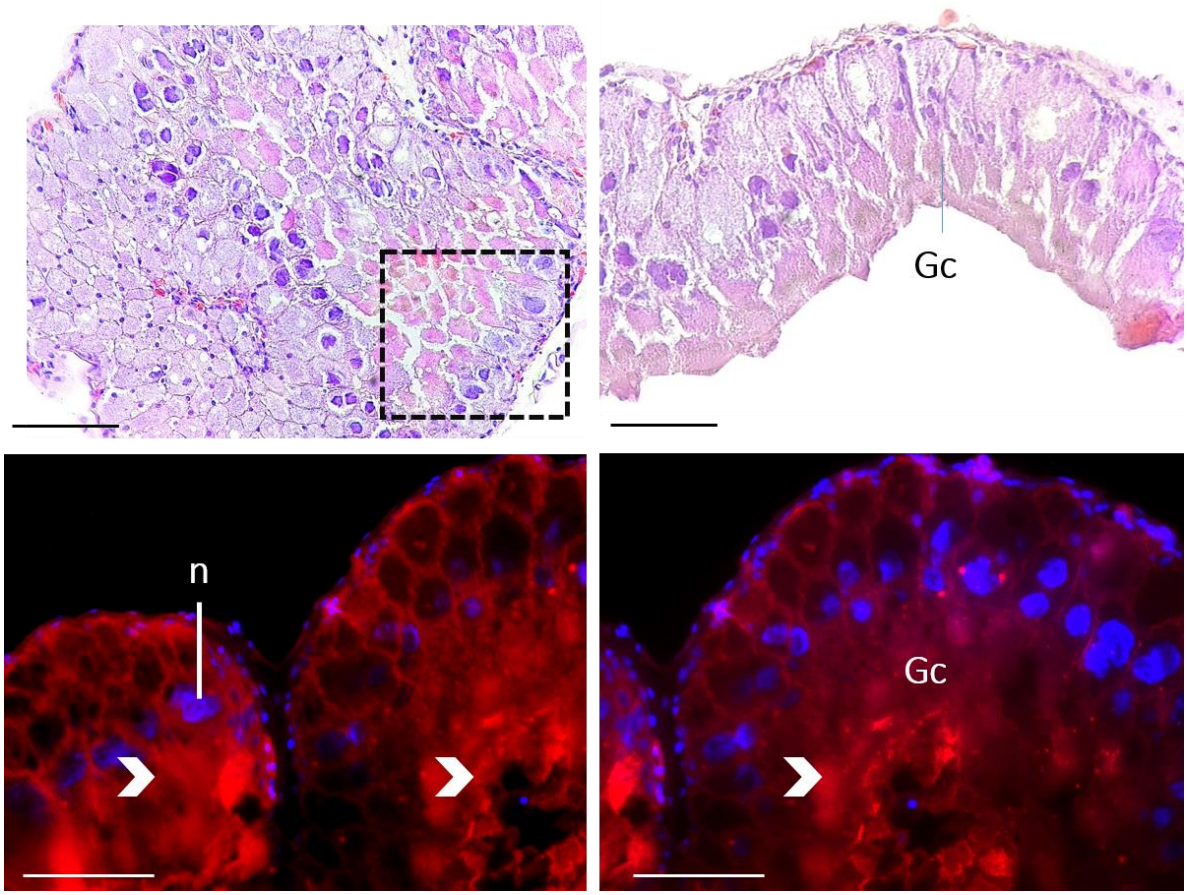

Figure 5 Histological sections of *Mabuya* sp placental tissues at late development. CaBP-9K labels the giant cells in the chorionic epithelium of the placentome in the embryonic hemisphere. Upper images represent hematoxylin and eosin staining; lower images show CaBP-9K immunolabelling. Nuclei (blue) are dye with DAPI. Arrows indicate the strong positive labeling. Gc, giant cells; n, nucleus.

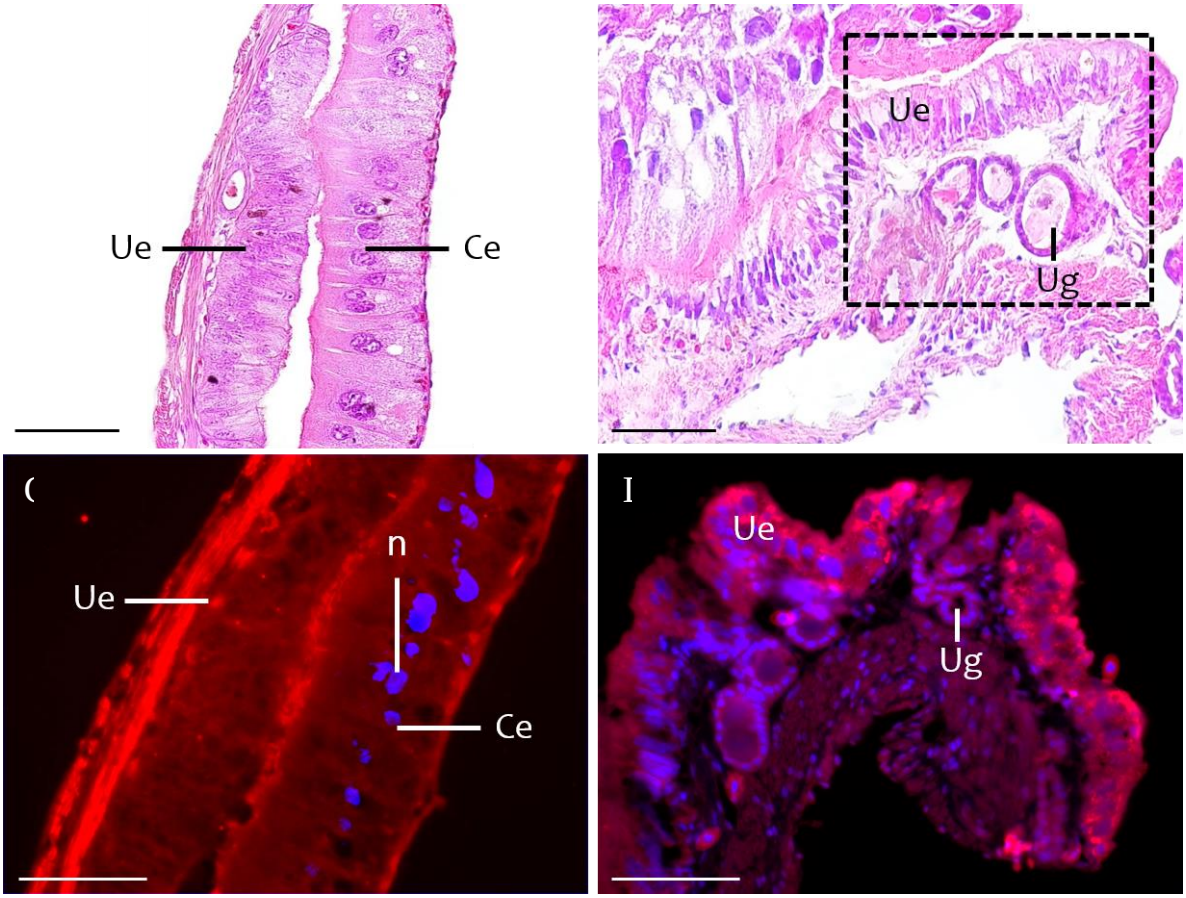

Figure 6 CaBP-9K expression in placental tissues of *Mabuya* sp at late development stages A. Histological section stained with hematoxylin and eosin (HE), showing an absorptive plaque in the abembryonic hemisphere. B. Histological section of the paraplacentome ut erine epithelium stained with HE. C. CaBP-9K strong immunolabeling of the basal membrane of the uterine and the chorionic epithelium of the absorptive plaques. D. CaBP-9K detection on the uterine epithelium, with none expression on the uterine glands. Ue, uterine epithelium; Ce, chorionic epithelium; Ug, uterine glands. Scale bar: 30µM.