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THE VESICLES OF *PHYLLOMEDUSA SAUVAGII* (ANURA: HYLIDAE) NEST

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Abstract. *Phyllomedusa sauvagii* lays its eggs surrounded by peculiar vesicles in nests made with tree leaves and, until now, the function of these vesicles was thought to be for maintaining moisture. The histomorphological and histochemical analysis of the oviduct of individuals in ovulatory period, and the study of the vesicles in the oviduct, ovisac and in the nest, showed unexpected results. Vesicles originate from glycoconjugates, proteins and lipids secreted in the PCP and PC and organized in the oviductal lumen and, according to their content, it is possible to recognize at last four types. Furthermore, both in the ovisac and in the nest, vesicles show peculiar relationships with oocites/eggs/embryos through specific communication channels, showing the existence of more complex interactions than previously thought.

Keywords. *Phyllomedusa sauvagii,* ovipositiont, vesicles, histochemistry.

Introduction

Since the description of the nest of *Phyllomedusa iheringhi* by Ihering (1886), the oviposition mode in that genus attracted considerable attention. Even in the last decades of the 19th Century, the fact that "… *Phyllomedusa* does not lay its eggs in the water, although the larva develops in that element, but in the open air, in masses 40-50 millim. long by 15-20 broad, between leaves hanging over the water…" (Ihering, 1886) was a surprising fact, although Boulenger (1886) tried to minimize the discovery, remarking, erroneously, that "… the fact observed [by Ihering] is not new among frogs. Another arboreal form *par excellence, Chiromantis rufescens,* Gthr. (= *C. guineensis* Buchh. and Ptrs.), from West Africa, … deposits its eggs in a similar way, as we know from a note published by Buchholz…", referring to the comments of Buchholz in Peters (1875).

The earlier contributions on this subject either did not mention the presence of jelly among the unpigmented eggs (Ihering, 1886), or just that "… the jelly in which the eggs were laid was of sufficient firmness to hold the edges of the leaf together…" (Budgett, 1899). Agar (1909) was the first to note that the jelly that surrounded the eggs in the nest of *Phyllomedusa sauvagii* was structured in spherical, transparent, capsules and, since then, almost all authors have mentioned the presence of this jelly in nests of several different species (*i.a.* Giaretta *et al.,* 2007, *Phyllomedusa araguari;* Block *et al.,* 2002, Duellman *et al.,* 1988, *Phyllomedusa atelopoides;* Budgett, 1899, Rodrigues *et al.,* 2008, *Phyllomedusa azurea;* Lescure *et al.,* 1995, *Phyllomedusa bicolor;* Vaira, 2001, *Phyllomedusa boliviana;* Abrunhosa and Wogel, 2004, *Phyllomedusa burmeisteri;* Prado *et al.* 2006, Castanho, 1994 (not seen), *Phyllomedusa distincta;* Lescure *et al.,* 1995, Matos *et al.,* 2000, Pyburn, 1980, Pyburn and Glidewell, 1971, *Phyllomedusa hypochondrialis;* De Sa and Gerhau, 1984, Klappenbach, 1961, Langone, 1993, Langone *et al.,* 1985, *Phyllomedusa iheringi;* Barrio-Amoros, 2006, *Phyllomedusa neildi;* Wogel *et al.,* 2005, *Phyllomedusa rohdei;* Agar, 1909, Rodrigues *et al.,* 2008, *Phyllomedusa sauvagii;* Lescure *et al.,* 1995, *Phyllomedusa tomopterna;* Kenny, 1966; 1968, *Phyllomedusa trinitatis;* Lescure *et al.,* 1995, *Phyllomedusa vaillantii*).

Almost all authors agree that the jelly spheres serve to glue the leaves of the nest after amplexus and to protect the eggs from desiccation, but the observations of this unique character among anurans usually stopped at the "field notes" stage. Among the few exceptions to the previous statement, Pyburn's (1980) contribution is the only one that tested experimentally the role of the eggless capsules in the survival of the embryos of *Phyllomedusa hypochondrialis,* while that of Agar (1909) deserves a special mention. A century ago, and after the dissection of two females of *Phyllomedusa sauvagii,* Agar stated: "… The question of how rounded egg-capsules are formed without egg as nuclei to form round, is one to which I have not found any clue by the dissection of the oviducts, which were nearly empty in both females I opened. I can only say that I found empty capsules far up in the glandular portion of the oviduct, as well marked off from one another as in the ovisacs…". Up to now, Agar's question remains unanswered.

In this contribution, we analyze the origin, histology and histochemistry of the eggless, gelatinous vesicles associated to the eggs in *Phyllomedusa sauvagii.*

Material and Methods

Gravid females of *Phyllomedusa sauvagii* were collected in diverse localities of Tucumán Province, in Northern Argentina. Voucher specimens were housed in the collection of the Instituto de Herpetología, while the histological slides were housed at the Instituto de Morfología Animal, both at Fundación Miguel Lillo, Tucumán, Argentina (*see* Appendix).

The histological and hystochemical analysis focused on oviducts of gravid females during the preovulatory and ovulatory stages, as well as immediately after spawning, and on the vesicles and eggs from the nests, to analyze the relationships between oocytes or eggs and the capsules.

The oviducts were divided into five grossly observable portions [in cephalic-caudal direction, *Pars Recta* (PR), Intermediate Proximal Zone (IPZ), Preconvolute Part (PCP), Convolute Part (CP) and Ovisacs (O)]. Samples were fixed for 24 hours in an isotonic and buffered formaldehyde solution [formaldehyde 10% in phosphate buffer (0.2 M to pH 7.2)], and in Stieve (saturated solution of mercury chloride 38 ml, acetic acid 2 ml, pure formaldehyde 10 ml). Finally, due to the characteristics of the vitellus, the samples from the ovulatory stage were treated with butylic acetate, n-butylic alcohol and/or Dioxane. All the samples were embedded in Paraplast and cut into serial and semi-serial sections of 4 to 7 µm.

The samples for histomorphological analysis were stained with Hematoxilin-Eosin (H-E), and Mallory (Azán) and Heindenhein (MA) Trichromics, while those for histochemistry were treated with the following staining solutions:

Periodic Acid-Schiff (PAS): Three intensities were recognized, indicating the presence of glycosidic linkages 1, 2-glycol:

- PAS+: biomolecules of low molecular weight, neutral glycolipids, and glycoproteins. These biomolecules had scarce oligosaccharide residues, a majority of sialic acid residues and low amount of hexosamines.
- PAS++: esterified mucoproteins with few acid residues and cerebrosid and ganglioside glicolipids.
- PAS+++: mucoproteins and proteoglycans highly polymerized, neutral and with some acid residues generally carboxilated.

Alcian Blue 8 GX (AB) at pH 2.5 and pH 0.5: Employed for the identification of glycosaminoglicans (GAG) and proteoglycans with carboxyl, phosphated, sialilated and sulfated compounds as terminal groups. At pH 0.5 sulfated and sialilated mucosubstances are evident.

Hale-Müller (Hale, 1946): To confirm the presence of glycoconjugates with carboxyl and some sulfated groups.

Alcian Blue pH 2.5-0.5 and Hale-Müller-PAS: this combination (Pearse, 1969) allows the differential identification of GAG and neutral proteoglycans, plus different acid groups.

Hematoxylin-Eosin: For the detection of glycoconjugates (glycoproteins and glycolipids, peptidoglycans, proteoglycans and lipoproteins).

Toluidine Blue, pH 7.0-7.3; pH 5.6 and pH 2-3-3.5: To confirm the presence of the groups detected with AB at pH 0.5. Its alcohol-resistence and the various metachromatic conditions were considered:

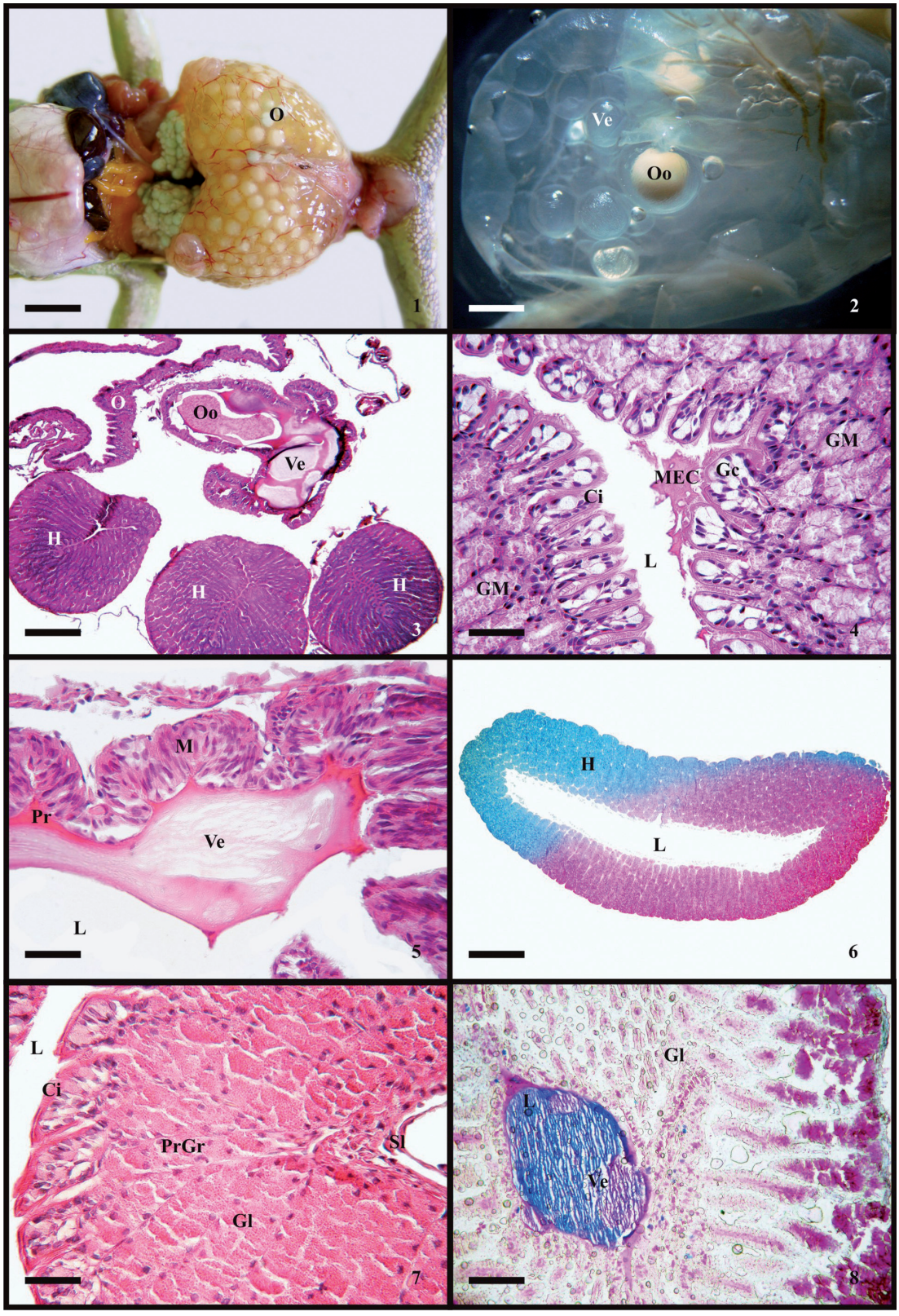
- α-metachromasy (orthochromasy): for the detection of basic proteins and links to negative groups and/or scarce and isolated ionic groups.
- β-metachromasy: alcohol non-resistent. For the detection of COOH and metaphosphate residues.
- γ-metachromasy: for the detection of glycoconjugates with sulphate and/or phosphate radicals.

The presence of the recorded compounds was double-checked through acetylation and methylation of the reactive groups and through the digestion with amylase, hyaluronidase and sialidase (Barka and Anderson, 1965; Pearse, 1960; Humason, 1979).

Results

General histomorphology of the oviduct

The structure of the oviduct of *Phyllomedusa sauvagii* is, in general, similar to that of other anurans, both in gross anatomy and histology (*see* Alcaide *et al.,* 2009, and literature contained therein). The particular characteristics of the oviduct of this species are located in the mucous, between the Preconvolute Part (PCP) and the last handle of the Convolute Part (CP). The epithelium of the mucosa of PCP shows (1) wide, uniformly distributed, folds, with the openings of the acinous-tubular glands between them;



Figures 1-8. 1. Ovisac *in situ.* 2. Ovisac. 2,0X, scale bar: 1 cm = 0.450 µm. 3. Oviduct. Hematoxilin-Eosin (H-E). 4X, scale bar: 1 cm = 0,240 µm. 4. Oviduct PCP. Hematoxilin-Eosin (H-E). 40X, scale bar: 1 cm = 0,023 µm. 5. Ovisac. Hematoxilin-Eosin (H-E). 40X, scale bar: 1 cm = 0,023 µm. 6. Oviduct PCP. Alcian Blue (AB) pH 2.5 – Periodic Acid-Schiff. 4X, scale bar: 1 cm = 0,240 µm. 7. PCP – PC. Hematoxilin-Eosin (H-E). 40X, scale bar: 1 cm = 0,023 µm. 8. PCP distal. Alcian Blue (AB) pH 0.5 – Periodic Acid-Schiff. 40X, scale bar: 1 cm = 0,023 µm. References: PCP: preconvolute pars, CP: convolute pars, Ov: oviduct, H: handle, Gc: goblet cells, O: ovisac, L: lumen, Ci: cilia, Oo: oocyte, Li: lipids, Pr: proteins, Ch: channel, Ve: vesicle, Gl: glands, MEC: matrix extra cell, M: mucous, PrGr: proteic granules, Sl: serosa layers, C: cover, Yp: yolk platelets, GM: mucous glands, GAG: glicosaaminoglicans. H: handle.

(2) voluminous goblets cells that contain a mosaic of biomolecules, and (3) mucous glands containing polymerized mucoproteins of high molecular weight (Figs. 4 and 5).

In the last handles of the PCP a transition of contents in the goblet cell and glands is evident due to the presence of glycoconjugates (GAG) with phosphated, carboxilated and/or sialilated radicals, and glandular mosaics of acid GAG and proteins. The glandular cells in all handles of the CP have proteic granules (PrGr) in their cytoplasm. (Figs. 6, 7).

The mucous of the last handle of the CP shows the transition between the typical structure of the oviduct and that of the ovisac (O), evidentiated by loose, deep folds with cylindrical-ciliated, secretory cells, a highly developed chorion, rich in collagen fibers, fibroblasts, mast cells, and neuro-vascular bundles. The serosa layer is highly innervated and has macroscopically visible vessels with a significant diameter,. Finally, during the ovulatory period, the ovisac in *Phyllomedusa sauvagii* looks like a highly distended sac, with the mucosa showing deep, wide and loose folds, and sulphated GAG (alcohol-resistant metachromasy) in its lining cells; the chorion is greatly developed, resistent and rich in collagen, while the serosa is thick, with several layers of muscular and collagen fibers. (Fig. 3).

Histochemical aspects and the origin of the vesicles

The formation of the vesicles starts in the cells of the glands of the proximal region of the Preconvolute Part (PCP) of the oviduct. The first step consists in the synthesis and secretion of mucoproteins to the glandular lumen. These contents are later released to the oviductal lumen, and from this point, and along the following two-thirds of the PCP, the secretory cells of the oviductal handles synthesize and release protoglucans with carboxyled and phosphated radicals. Due to the action of the ciliated epithelial cells and, probably, to the peculiarities of the intracellular cytoskeleton (Albert *et al.,* 2004), there are interactions among complex (both in contents and structure) biomolecules, resulting in vesicles with organized, acid and neutral glycoconjugates, that constitute the vesicles core. (Fig. 8).

Protein synthesis starts in the distal third of the PCP. under the presence of acid glycosaminoglycans (GAG). Proteins are released as coalescing granules that, in the oviductal lumen, conform the proteic cover of the vesicles. This process continues in the Convolute Part (CP), where, aside from the protein-secreting glands, there are goblet cells with three types of content, including neutral and acid GAG and a combination of both types. The last handles of the CP show a transitional aspect to the ovisac (O), and at that point there are no contributions to the structure of the vesicles. (Fig. 3).

Oocytes and vesicles in the ovisac

During the ovulatory period, oocytes and vesicles have a perfectly uniform, not random, distribution in the ovisac. The proximal region of each ovisac is filled only with vesicles, the medial region shows a mixture of vesicles and oocytes, and the contact area between ovisac and cloaca has, again, only vesicles. (Figs. 1, 2).

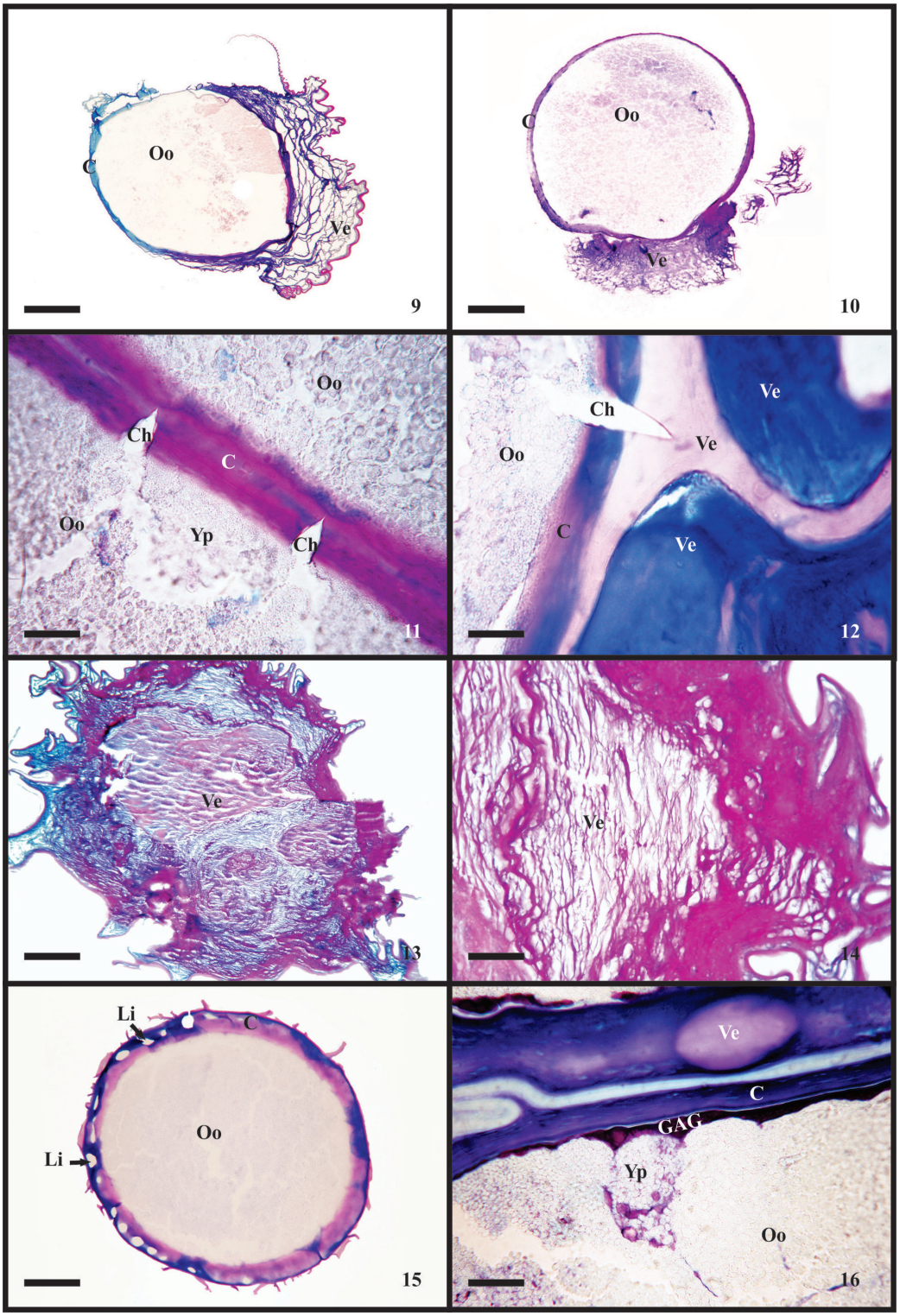
The vesicles of the medial region of the ovisac are in intimate contact between one another and with the oocytes (Figs. 9, 10). In the last case, both elements have extremely flexible covers of proteic nature forming channels that run from the vesicles to the oocyte, penetrating to the cortical region after the rupture of the vitelline envelope. Our observations suggest that the oocyte has specific areas for the dehiscence and rupture of the cover, evidentiated by the clear and well-defined separation of the cortical granules and the structure of the surrounding covers. This contact, in the form of a finger-like fold, clearly demonstrates the transition of the heterogeneous contents of the vesicles to the oocytes (Figs. 11, 12).

A noticeable trait in the ovisac in this period is the complete lack of accessory, amorphous jelly.

Oocytes and eggless vesicles in the nest

The aerial nest of *Phyllomedusa sauvagii* contains a mixture of vesicles and eggs, distributed in a way coincident with what was described for the ovisac. The upper and lower parts of the nest contain only vesicles, surrounding a medial area with oocytes and vesicles.

The vesicles are hydrated and the structure is modified in relation to those in the ovisac, mainly visible in the folds of the proteic cover and in the fibrilar organization of its biomolecules. In general, the peripheral fibers are loosely distributed, while the inner ones have a whirling aspect, conforming a central, compact core. From the histochemical standpoint, there are three types of content in the capsules,



Figures 9-16. 9. Nest. Oocyte – Vesicle. Alcian Blue (AB) pH 2.5 – Periodic Acid-Schiff. 10X, scale bar: 1 cm = 0,095 µm. 10. Nest. Oocyte – Vesicle. Alcian Blue (AB) pH 0.5 – Periodic Acid-Schiff. 10X, scale bar: 1 cm = 0,095 µm. 11. Nest. Oocyte – Vesicle – Cover. Alcian Blue (AB) pH 0.5 – Periodic Acid-Schiff. 63X, scale bar: 1 cm = 0,014 µm. 12. Nest. Oocyte – Vesicle – Cover. Alcian Blue (AB) pH 2.5 – Periodic Acid-Schiff. 63X, scale bar: 1 cm = 0,014 µm. 13. Nest. Oocyte – Vesicle. Alcian Blue (AB) pH 2.5 – Periodic Acid-Schiff. 40X, scale bar: 1 cm = 0,023 µm. 14. Nest. Oocyte – Vesicle. Alcian Blue (AB) pH 2.5 – Periodic Acid-Schiff. 63X, scale bar: 1 cm = 0,014 µm. 15. Nest. Oocyte – Vesicle – Cover. Alcian Blue (AB) pH 2.5 – Periodic Acid-Schiff. 10X, scale bar: 1 cm = 0,095 µm. 16. Nest. Oocyte – Vesicle – Cover. Toluidin Blue (TB) pH 3,5. 40X, scale bar: 1 cm = 0,023 µm. References: PCP: preconvolute pars, CP: convolute pars, Ov: oviduct, H: handle, Gc: goblet cells, O: ovisac, L: lumen, Ci: cilia, Oo: oocyte, Li: lipids, Pr: proteins, Ch: channel, Ve: vesicle, Gl: glands, MEC: matrix extra cell, M: mucous, PrGr: proteic granules, Sl: serosa layers, C: cover, Yp: yolk platelets, GM: mucous glands, GAG: glicosaaminoglicans. H: handle.

including those with lipids, with highly polimerized mucoproteins, or with acid GAG. (Figs. 13, 14, 15).

While the vesicles/egg relationship seems to be determined by structural peculiarities of the latter, the vesicles/vesicles contact seems to occur haphazardly. An interesting phenomenon is that the vesicles content will be released into the egg either directly (in the vesicles/egg case) or through intermediate vesicles (in the capsule/capsule case). The content of the vesicles, of a gelatinous aspect, surrounds the eggs as a thin layer of groups of yolk platelets, and is also present as a central part of the structure thus formed (Fig. 16).

Discussion

Pyburn (1980) considered that the vesicles found in nests of *Phyllomedusa* were structures filled with metabolic water that had a key role in maintaining a proper humid environment for the development of eggs and embryos. Our observations show that in addition to metabolic water, the vesicles contains proteoglycans with different reactive groups (acids and neutral), plus lipidic compounds in vacuoles and hydorphilic proteins that modify their structure depending on the degree of hydration. We also saw that the contents of the vesicles penetrate the oocytes by intra-oocyte channels, demonstrating the existence of complex relationships between the vesicles and the oocytes, which are pivotal to the development of the embryo.

As already pointed out, the vesicles are formed in the PCP-CP area of the oviduct, while the mucin and lipid contents are produced by cells in the mucosa of different loops of the PCP, and the proteins are contributed by cells of the PC. These two areas, with such different contents, have not been observed in other studied species.

The ovisac also has a peculiar structure, with very thick folds coated with cylindrical, epithelial cells containing sulfated mucoproteins (and alcohol-resistant metachromasia), and the chorion has a fibrillar nature (collagen and some muscle fibers). This is a key character to enable distension and therefore accommodate numerous oocytes and vesicles, in a ratio of about 1:10 +. In the ovisac the presence of vascular-nervous packages is also noticeable, with macroscopically observable medium-calibre vessels, an image that allows us to infer the control of the hypothalamic pituitary at the time of spawning.

Another interesting fact is that in *Phyllomedusa sauvagii* there is no accessory jelly as reported in other species of amphibians (Alcaide, 2006), so we conclude that the vesicles would be homologous to that substance.

Finally, the distribution of vesicles and eggs in the nest corresponds to that observed in the ovisac, and is coincident with those reported by Adler (1910). It is also important to note the conformational change of the vesicles in the nest, as a result of hydration and the already mentioned interactions, with the consequent formation of channels structured by the oocyte cover and the vitelline membrane, with the displacement of cortical granules and the incorporation of the vesicle's contents.

Resumen

Phyllomedusa sauvagii deposita sus huevos rodeados de vesículas peculiares en nidos hechos con hojas de árbol y, hasta ahora, se pensaba que la función de dichas vesículas era mantener la humedad de la puesta. Análisis histomorfológicos e histoquímicoss del oviducto en período ovulatorio, así como el estudio de las vesículas en oviducto, ovisaco y nido, mostraron resultados inesperados. Las vesículas se originan a partir de glicoconjugados, proteínas y lípidos secretados en PCP y PC y organizados en el lumen del oviducto y, de acuerdo con su contenido, es posible reconocer a cuatro tipos. Por otra parte, tanto en el ovisaco como en el nido las vesículas muestran relaciones peculiares con los ovocitos/huevos/embriones a través de canales de comunicación específicos, mostrando la existencia de interacciones más complejas de lo que se pensaba.

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Appendix

Phyllomedusa sauvagii

Number of personal register	Number of collection (FML – MRM – MCN)	Sexual period	Location
AP 5	FML 7738	Ovulatory	El Ceibal – Tucumán
AP 4	FML 23830	Ovulatory	Horco Molle – Tucuman
AP 8	MRN 0014	Ovulatory	Horco Molle – Tucuman
AP 9	MCN 883	Ovulatory	Salta