The Adhesive Glands during Embryogenesis in Some Species of Phyllomedusinae (Anura: Hylidae)

Author(s): Florencia Vera Candioti, Carlos Taboada, María José Salica, Diego Baldo, Julián Faivovich, and Délio Baêta

Source: Journal of Herpetology, 51(1):119-129.
Published By: The Society for the Study of Amphibians and Reptiles
DOI: http://dx.doi.org/10.1670/15-127
URL: http://www.bioone.org/doi/full/10.1670/15-127

Journal of Herpetology, Vol. 51, No. 1, 119–129, 2017

The Adhesive Glands during Embryogenesis in Some Species of Phyllomedusinae (Anura: Hylidae)

Florencia Vera Candioti,[1,2] Carlos Taboada,[3] María José Salica,[4] Diego Baldo,[5] Julián Faivovich,[3,6] and Délio Baêta[7,8]

[1]*Instituto de Herpetología, Unidad Ejecutora Lillo CONICET–FML, San Miguel de Tucumán, Argentina*
[3]*División Herpetología, Museo Argentino de Ciencias Naturales "Bernardino Rivadavia"–CONICET, Buenos Aires, Argentina*
[4]*Centro de Investigación y Transferencia de Jujuy CONICET–Universidad Nacional de Jujuy, San Salvador de Jujuy, Argentina*
[5]*Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical CONICET–UNaM, Posadas, Argentina*
[6]*Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina*
[7]*Laboratório de Herpetologia, Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, Rio Claro, Brazil*
[8]*Herpetologia, Departamento de Vertebrados, Museu Nacional, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*

Abstract. **Among anuran embryonic structures, the adhesive (cement) glands appear posterolaterally to the stomodeum and produce a mucous secretion that adheres embryos to surfaces in and out of the egg. In this paper, we study the ontogeny of the adhesive glands in five species of *Phyllomedusa* representing the two main clades recognized in the genus, plus embryos of *Agalychnis aspera* and *Phasmahyla cochranae*. Clutches were collected in the field, and embryos were periodically fixed to obtain complete developmental series and then studied with a stereomicroscope, scanning electron microscopy and routine histological techniques. Structural variations include glands absent (in *P. cochranae* and *Phyllomedusa boliviana*), functional club-shaped glands (morphogenetic Type C in *Phyllomedusa sauvagii*, *Phyllomedusa iheringii*, and *Phyllomedusa tetraploidea*), and an unusual Type C-like pattern in *Phyllomedusa azurea*, characterized by large, oblong glands in a horseshoe-like disposition around the oral disc. This latter gland configuration is similar to that of *A. aspera*. Interspecific variations also include the arrangement and regression pattern of the secretory region, which are in turn different from those of Type C glands in other clades. To interpret the origin and evolution of gland developmental patterns in the group, we still need information on gland occurrence and development in the basal genera of Phyllomedusinae (*Phrynomedusa* and *Cruziohyla*) and in the basal taxa of the two major clades of *Phyllomedusa*.**

Resumen. **Entre las estructuras embrionarias de los anuros, las glándulas adhesivas (o de cemento) aparecen posterolaterales al estomodeo y producen una secreción mucosa que adhiere los embriones a las superficies dentro y fuera del huevo. En este trabajo estudiamos la ontogenia de las glándulas adhesivas de cinco especies de *Phyllomedusa*, representantes de los dos principales clados reconocidos en el género, y de embriones de *Agalychnis aspera* y *Phasmahyla cochranae*. Las puestas se colectaron en el campo, los embriones fueron fijados periódicamente para obtener series de desarrollo completas y luego estudiados con lupa estereoscópica, microscopía electrónica de barrido y técnicas histológicas de rutina. Las variaciones estructurales incluyen glándulas ausentes (en *P. cochranae* y *Phyllomedusa boliviana*), glándulas cónicas funcionales (Tipo morfogenético C, en *Phyllomedusa sauvagii*, *Phyllomedusa iheringii* y *Phyllomedusa tetraploidea*), y un patrón similar al Tipo C inusual en *P. azurea*, caracterizado por ser glándulas grandes, oblongas, con una disposición en herradura en torno al disco oral. Este último patrón es comparable al observado en *A. aspera*. Las variaciones interespecíficas también conciernen al arreglo y patrón de regresión de la región secretora, a su vez diferentes del de glándulas Tipo C de otros clados. Aún se requiere información sobre la ocurrencia y desarrollo de las glándulas en géneros basales de Phyllomedusinae (*Phrynomedusa* y *Cruziohyla*), y en los taxones basales de los dos clados principales de *Phyllomedusa*, a fin de interpretar el origen y evolución de los patrones morfogenéticos de las glándulas adhesivas en el grupo.**

Studies on comparative developmental patterns, including early stages during embryogeny, have gained interest in evolutionary biology as they allow us to elucidate how changes in development can produce morphological changes in adults and also to explore structural and temporal variations at those poorly known periods. Anuran early ontogeny includes the development of transient structures that do not persist in larval stages but disappear around hatching (Nokhbatolfoghahai and Downie, 2005, 2007, 2008; Nokhbatolfoghahai et al., 2005, 2006). Among transient embryonic structures, the adhesive (cement) glands are secreting organs formed of ectodermic bordering cells and secretory cells. They are absent in most anurans with endotrophic development (e.g., species with direct development), along with several embryonic and larval features. In species with exotrophic development, glands appear posterolaterally to the stomodeum and produce a mucous secretion that adheres anuran embryos to surfaces in and out of the egg (Nokhbatolfoghahai and Downie, 2005). In *Xenopus laevis* embryos, Bles (1905) suggested these structures are physically involved during hatching, by anchoring the head on the inner vitelline membrane surface and then allowing a local softening of this membrane through secretion of hatching cells. In general, glands have their maximum development around hatching and regress by the time hind limbs emerge. The morphology and development of these glands were studied in detail for *X. laevis* (e.g., Wardle and Sive, 2003) and comparatively in a few species (e.g., *Bufo* spp., *Rana* spp.; Pennati et al., 2000). Nokhbatolfoghahai and Downie (2005) described anuran embryos of 20 species and characterized these glands for six families. Among hylids, variations include glands where an initial V-shaped groove forms to two oval structures or glands composed of two club-shaped structures that separate early (called types A and C, respectively, by Nokhbatolfoghahai and Downie, 2005).

The Phyllomedusinae are a group of mainly arboreal frogs distributed from Mexico to Argentina (Frost, 2015). From a developmental point of view, they are an interesting group to study because of the peculiar reproductive biology (including

[2]Corresponding Author. E-mail: florivc@gmail.com
DOI: 10.1670/15-127

different sites of terrestrial oviposition and in several species bladder filling behavior, leaf folding behavior, and eggless capsules in clutches), late hatching, and extensive variation in larval morphology and ecology (Faivovich et al., 2010). The embryonic ontogeny has been described for only a few species in the subfamily, including four species of *Phyllomedusa* (Budgett, 1899; Lutz and Lutz, 1939; Kenny, 1968; Salica et al., 2011) and two of *Agalychnis* (Pyburn, 1963; Vargas and Gutiérrez, 2005). Studies in this latter genus, and more recently also in embryos of *Phyllomedusa trinitatis*, are mostly made within an ecological, experimental context, in relation to the ability these embryos have to hatch early in response to external stimuli. The other genera of phyllomedusines are comparatively less known, and information on the initial development of the unusual, neustonic tadpoles of *Phasmahyla*, with their upturned umbelliform oral discs (e.g., Altig and McDiarmid, 1999), is not available.

In this paper, we study the ontogeny of adhesive glands in five species of *Phyllomedusa* representing the two main clades recognized in the genus by Faivovich et al. (2010). For comparative purposes, we also examine embryos of *Phasmahyla cochranae* and *Agalychnis aspera* and contrast our results with previous works to discuss morphological and ontogenetic variation of these structures in a phylogenetic context.

Materials and Methods

We studied series of early development of *A. aspera* (Peters, 1873), *P. cochranae* (Bokermann, 1966), *Phyllomedusa azurea* Cope, 1862, *Phyllomedusa boliviana* Boulenger, 1902, *Phyllomedusa sauvagii* Boulenger, 1882, *Phyllomedusa iheringii* Boulenger, 1885, and *Phyllomedusa tetraploidea* Pombal and Haddad, 1992. Series were obtained from clutches collected in the field (Table 1). The leaf nests were taken to the lab and hung at the walls of glass containers filled with tap water, above the level of the water; the clutches were kept in ambient room temperature and natural light cycle. We carefully removed an average of 6 eggs from each nest every 8–12 hours until hind limbs emerged and then euthanized the embryos with MS222 anesthetic and preserved them in 10% formalin. Embryos were categorized following the stages of early development described for *P. trinitatis* (Kenny, 1968; abbreviated here as K); because gland regression co-occurs with hind-limb emergence, we used Gosner's (1960; G) table to categorize older embryos starting in Gosner stage 25 (that is, after K24). The external morphology of the specimens was first studied with a stereomicroscope, staining with methylene blue for better contrast. Summarized series of developmental stages ($N = 3$–12) for each species were dehydrated with an ascending series of ethanol and later critical point dried and coated with gold for analysis with scanning electron microscopy.

For histological studies, three formalin-fixed specimens of *P. azurea*, *P. iheringii*, and *P. sauvagii* at K23 were dehydrated in an ascending series of ethanol, cleared in toluene, and embedded in Paraplast. Transverse sections were sectioned from the head of the tadpoles at 5 μm each and then stained with hematoxylin and eosin for general topography and a combined Alcian blue-PAS procedure (Mowry, 1956) for acid and neutral mucosubstances, respectively. Terminology used for descriptions is that of Picard (1976) and Altig (2007). For comparison of adhesive gland morphogenetic patterns, we also analyzed embryos of other hylid taxa (i.e., *Pseudis minuta*, *Dendropsophus* spp., and *Scinax* spp., detailed in Appendix 1).

Results

Phyllomedusine embryos vary in presence of adhesive glands. In those species with glands, structural patterns vary mainly in gross morphology. Additionally, heterochronic differences are recorded at the developmental stage where the glands are completely regressed (Table 1).

In *P. sauvagii*, *P. iheringii*, and *P. tetraploidea*, adhesive glands are first visible at K18–19, as a curved prominence that rapidly (about K19/20) divides into two rounded bulges posterior to the stomodeum. At K22 (Fig. 1A, C, E), they reach their maximum development and have a conical shape with a somewhat depressed central region at the apex. Two superficial cell types are distinguished (Fig. 1B, D, F and 2B, D). The gland is completely bordered by a planar ectoderm formed of bordering cells with surface microridges or cilia; ciliated cells are scarce in this region as compared to other surfaces of the embryo. Secretory cells are congregated at the top of the gland; they comprise a pseudostratified columnar epithelium that is covered by the bordering cells, except for the eosinophilic apices that protrude slightly above the level of lateral contacts and present a distinct striated border (Fig. 2B). Secretory cells are highly elongated, and the cytoplasm presents two easily differentiated zones. The apical, supranuclear region presents a strong PAS positive secretory material and sparse pigments; the basal or subnuclear region contains several clear spaces, probably lipid droplets. Finally, below the secretory layer lies a basal layer of ectoderm composed of cuboidal basal cells. In *P. iheringii*, bordering cells overlap a large extension of the secretory cells, whose bulging apical surfaces are congregated in several, discontinuous patches at the top of the gland (Figs. 1D and 2D). The glands start to regress at K23, and regression is first evidenced by an overall loose of prominence. Next, the secretory region appears discontinuous (e.g., inset in Fig. 1B) until secretory cells disappear. As the glands regress they become lateral and finally anterolateral to the oral disc, such that remaining secretory cells are seen next to the upper lip (Fig. 6A–C). In *P. iheringii*, there remain some scattered secretory cells when the external gills are almost concealed, whereas in *P. tetraploidea*, a continuous, well-developed secretory region is still seen at this same stage. Glands are completely regressed after the spiracle is completely developed (in *P. iheringii* and *P. tetraploidea*) and after the hind-limb buds are evident (in *P. sauvagii*) (Table 1).

Adhesive glands of *P. azurea* embryos differ widely in gross morphology and development. They are first visible at K18/19, as a curved groove that rapidly at K19/20 (Fig. 3A) divides into two large, oblong structures posterior and lateral to the stomodeum; an elongated central groove is parallel to the long axis of each gland to which the apices of the secretory cells converge. At K22 (Fig. 3B), glands reach their maximum development, appearing almost contiguous posteriorly forming a horseshoe-like structure around the oral disc. Two superficial cell types (i.e., bordering cells with microridges or cilia and secretory cells with microvilli) are the same as in the former species (Figs. 3E–G and Fig. 4). Also, serial sections of the adjacent portions of the glands showed that, immediately posterior to them, there are groups of ellipsoid goblet-like, AB-positive cells displaying polarization with basal nucleus and apical cytoplasm (Fig. 4C). These cells are clearly differentiated from adjacent ectodermal cells, and some of them are both PAS and AB-positive resulting in a purple stained cytoplasm. After K23 (Figs. 3C, D), glands start to regress: the whole structure shrinks and later becomes less protruding and segmented (Fig.

TABLE 1. Material examined, with details of hatching stage, the stage when adhesive gland (AG) appears and disappears. Data on clutch size were taken from the literature, and number of eggs (average and range) and number of counted ovipositions (N) are consigned.

Species	Clutch size	Literature	Sample size*	Hatching stage	AG appears	AG disappears	Collection data
Agalychnis aspera	60, $N = 1$	Pimenta et al., 2007	16	later than K24**	not observed	later than K24	Brazil: Bahia: Porto Seguro, Reserva Particular do Patrimônio Natural Estação Veracel, CFBH 36905
Phasmahyla cochranae	$x = 35$ (21–48), $N = 27$	Sacramento et al., 2013	9	later than K24**	AG absent	AG absent	Brazil: Minas Gerais: Poços de Caldas, Morro do Ferro, CFBH 36940
Phyllomedusa azurea	$x = 333$ (77–766), $N = 3$; $x = 103$ (50–142), $N = 14$	Perotti, 1995; Rodrigues et al., 2007	~ 84	K23, K24	K18/19	G27	Argentina: Chaco: Wichi (24°41′30.2″S; 61°25′48″W), LGE 10645
Phyllomedusa boliviana	$x = 176$ (136–216), $N = 14$	Vaira, 2001	~ 68	K24	AG absent	AG absent	Argentina: Jujuy: Libertador General San Martín (23°46′S; 64°51′W), FML 28565
Phyllomedusa iheringii	72, $N = 1$	de Sá and Gerdau, 1983	~ 35	K24	K19/20	G25	Uruguay: Cerro Largo: Aceguá, LGE 10644
Phyllomedusa sauvagii	$x = 595$ (278–843), $N = 3$; $x = 594$ (201–829), $N = 15$	Perotti, 1995; Rodrigues et al., 2007	~ 87	K24	K18/19	G26	Argentina: Tucumán: Horco Molle (26°47′35″S; 65°19′00.2″W), FML 24189
Phyllomedusa tetraploidea	$x = 167$ (137–190), $N = 3$	Dias et al., 2013	~ 220	K24	K19/20	G25	Argentina: Misiones: Parque Provincial El Piñalito, Ruta Nacional No. 14 (26°25′S; 53°50′W) LGE 7728

*Only viable eggs and embryos are considered.
** All eggs were fixed before hatching.

3D). Secretory cells are no longer visible superficially and the whole region remains covered by a regular epidermis that progressively loses all ciliation. The region anterolateral to the oral disc is the last to regress (Figs. 3D and 6D). Glands are completely regressed when the hind limbs are as long as wide (about Gosner stage 27; Table 1).

Because of our small sample, we could describe only stages K22/24 of *Agalychnis aspera*. At the earliest stage, glands appear as two slightly protuberant regions with numerous, discontinuous patches of secretory cells with bulging apical surfaces and long microvilli, surrounded by polygonal bordering cells with microridges or cilia (Fig. 5A, B). The groups of secretory cells are placed bilaterally at commissures and posterolateral to the oral disc; in older embryos, groups become smaller but more widespread, reaching anterolateral corners of the disc and even its posteriormost region; hence, the whole structure loses its bilateral aspect. Regression occurs likely at the same time across the entire gland, and at K24 embryos, several small groups of secretory cells can still be seen anterolateral, lateral, and posterolateral to the disc (Fig. 6H). Finally, adhesive glands are absent in embryos of *P. boliviana* (Fig. 5C, D) and *P. cochranae* (Fig. 5E, F).

DISCUSSION

The presence and morphological configuration of adhesive glands of phyllomedusines vary interspecifically. Functional glands are absent in *Phasmahyla cochranae*, *P. boliviana* (this study), and *Phyllomedusa trinitatis* (Kenny, 1968; Nokhbatolfoghahai and Downie, 2005). Different morphological patterns have been described in other species. *Phyllomedusa sauvagii* and *Phyllomedusa rohdei* have small, conical glands (Lutz and Lutz, 1939; Salica et al., 2011), whereas *P. azurea* and *Agalychnis callidryas* show large, oblong glands in a horseshoe-like disposition around the oral disc (Pyburn, 1963; Warkentin, 1999; Salica et al., 2011). In addition, we report distinct, functional glands present in *P. iheringii*, *P. tetraploidea*, and *A. aspera*. The pattern in *P. iheringii* differs from that of *P. sauvagii* and *P. tetraploidea* because of the arrangement of the secretory region. In fact, this appears to be not a feature of the secretory cells but of the epidermal, bordering cells that cover most of the gland surface in *P. iheringii*, leaving small patches of secretory cell apices emerging instead of a single central secretory groove. Likewise, glands of *A. aspera* differ from those described in *A. callidryas* (see fig. 2 in Warkentin, 1999) in that the structures are only slightly prominent and show a discontinuous secretory region at the earliest stages analyzed.

The morphogenetic patterns of *P. sauvagii*, *P. iheringii*, and *P. tetraploidea* glands correspond with the Type C pattern defined by Nokhbatolfoghahai and Downie (2005), characterized by an initial groove that divides early (in our species, before the opening of the stomodeum) into two club-shaped glands posterolateral to the stomodeum. We interpret the pattern in *P. azurea* (and likely those of *Agalychnis* species) to also develop in a Type C-like way, where instead of small circular glands, two relatively large, oblong structures split from the initial groove. In all other types of gland development defined by Nokhbatolfoghahai and Downie (2005; excepting the Type E, exclusive of *Xenopus*), a V-shaped groove is formed and persists longer (at least until operculum differentiation) until the gland divides into two structures. A C-pattern is typical of hylids such as *Dendropsophus*, *Pseudis*, and *Scinax* (Nokhbatolfoghahai and

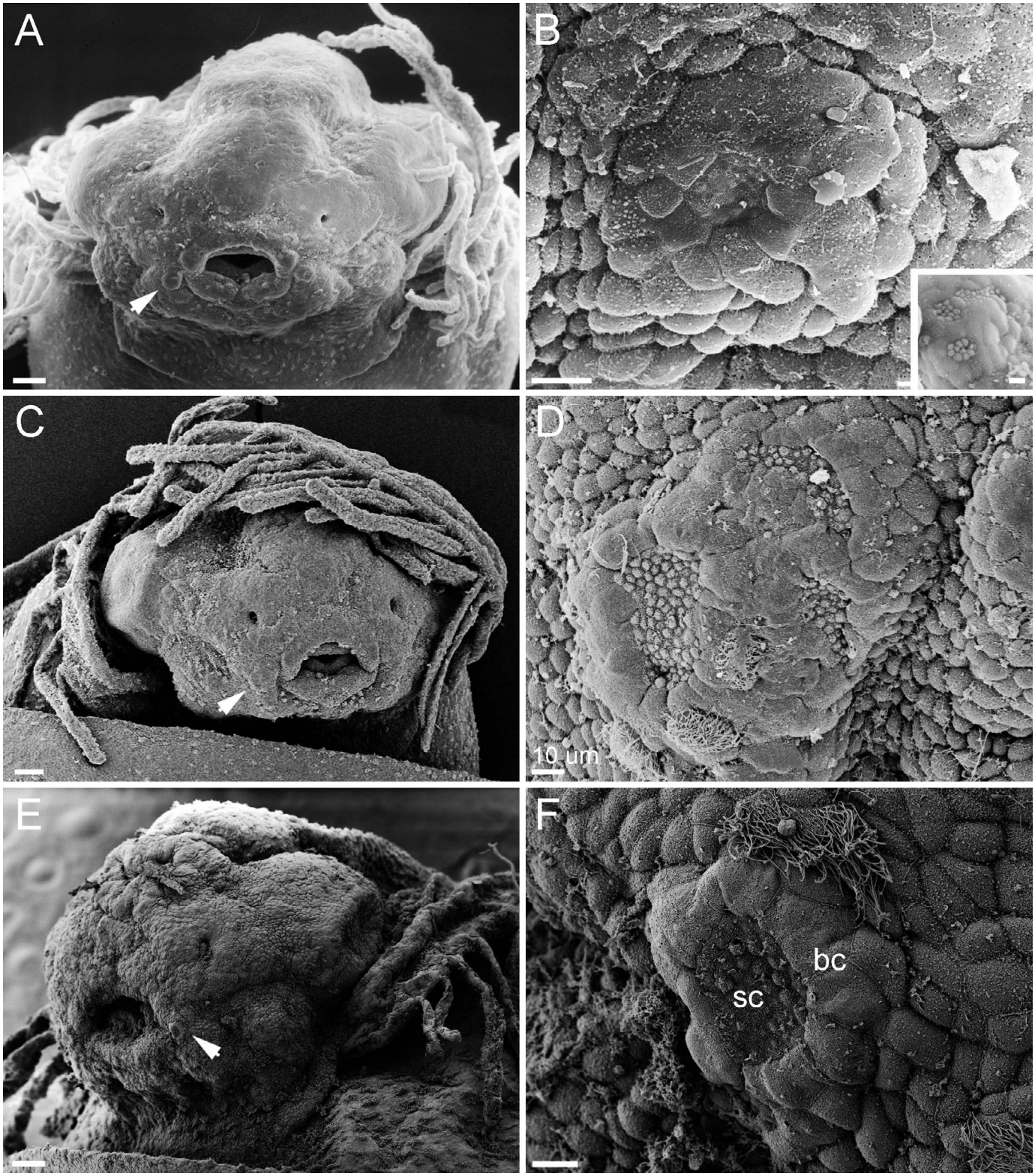

FIG. 1. Adhesive glands in embryos of *Phyllomedusa*, showing their placement lateral to the oral disc (arrows in A,C,E), and details of the superficial cell types. (A, B) *Phyllomedusa sauvagii* K22; the inset shows a more advanced, regressing gland at K24. (C, D) *Phyllomedusa iheringii* K22. (E, F) *Phyllomedusa tetraploidea* K21/22. Note the bordering cells (bc) and the secretory cells (sc) and the discontinuous arrangement of the secretory cells in the regressing gland of *P. sauvagii* and in the fully developed gland of *P. iheringii*. Scale lines = 100 μm (A, C, E) and 10 μm (B, D, F).

Downie, 2005; FVC unpubl. data), and it also occurs in several other clades of anurans (e.g., leiuperines, bufonids, microhylids, odontophrynids; Nokhbatolfoghahai and Downie, 2005; FVC unpubl. data).

Notwithstanding the differences in gland gross morphology, cell types described for adhesive glands in other anuran clades occur in *Phyllomedusa* species. Bordering ciliated cells are overall very scattered on and surrounding the glands, and they persist

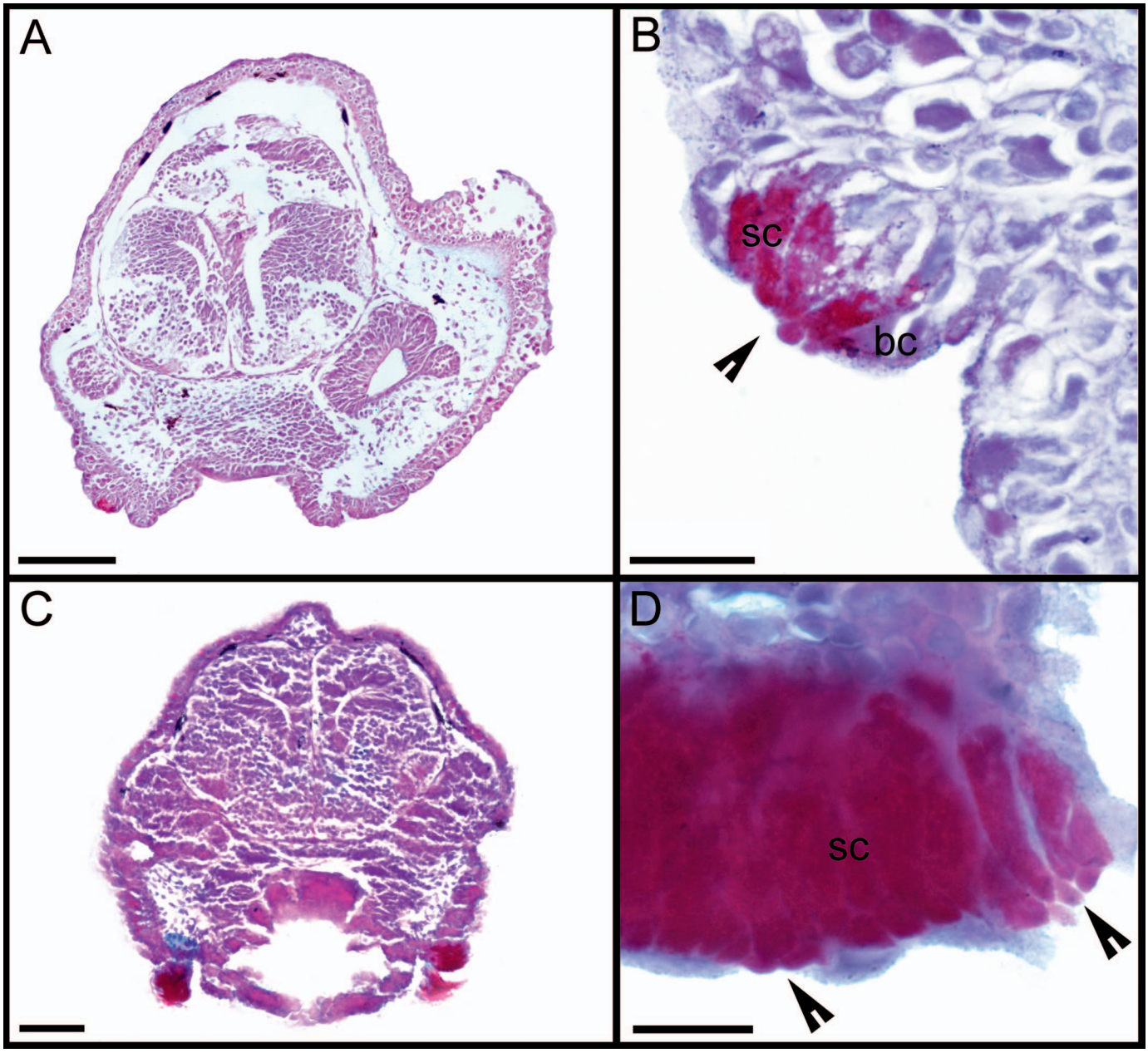

FIG. 2. Histological features of glands in embryos of *Phyllomedusa* at K23 (Alcian Blue-PAS), showing transverse, topographic views at the level of eyes, and details of secretory (sc) and bordering (bc) cells. (A, B) *Phyllomedusa sauvagii*. (C, D) *Phyllomedusa iheringii*. Note the patches of free apices of secretory cells (arrow tips), two in *P. iheringii*. Scale lines = 50 μm (A, C) and 2 μm (B, D).

approximately until K23 when neuromasts of the lateral lines develop. Low density of ciliation has been reported in *Dendropsophus minutus*, *Dendropsophus microcephalus*, *Dendropsophus minusculus*, *Engystomops pustulosus*, and *X. laevis* (all embryos with C-pattern glands except the latter; Nokhbatolfoghahai and Downie, 2005; Nokhbatolfoghahai et al., 2005). *Phyllomedusa azurea* also shows a fourth cell type clustered in the region immediately posterior to the adhesive glands. They are clearly distinguishable from elongated PAS positive cells and histochemically reveal acidic mucopolysaccharides content. Whether these cells are implicated in the adhesion mechanism or are involved in other biological functions should be further studied. Among species lacking glands, *P. trinitatis* embryos show paired, low bulges posterior to the oral disc, with no signs of differentiation in the ectodermal layer (Nokhbatolfoghahai and Downie, 2005). These bulges are not evident in *P. boliviana* in our study.

The ontogeny of glands is also similar among the species of *Phyllomedusa* that we studied. They appear about K19, posterolateral to the stomodeum, and reach their maximum development at K22, placed at the commissures (conical glands) or occupying an extensive area antero- and posterolateral to the oral disc (the horse-shoe arrangement). Regression starts by K23 approximately and is complete shortly after the appearance of hind limbs in all species but *P. azurea*, which still shows secretory cells with hind limbs as long as wide. The regression pattern is similar across the species: the whole structure first loses prominence, and then cell differentiation disappears. This

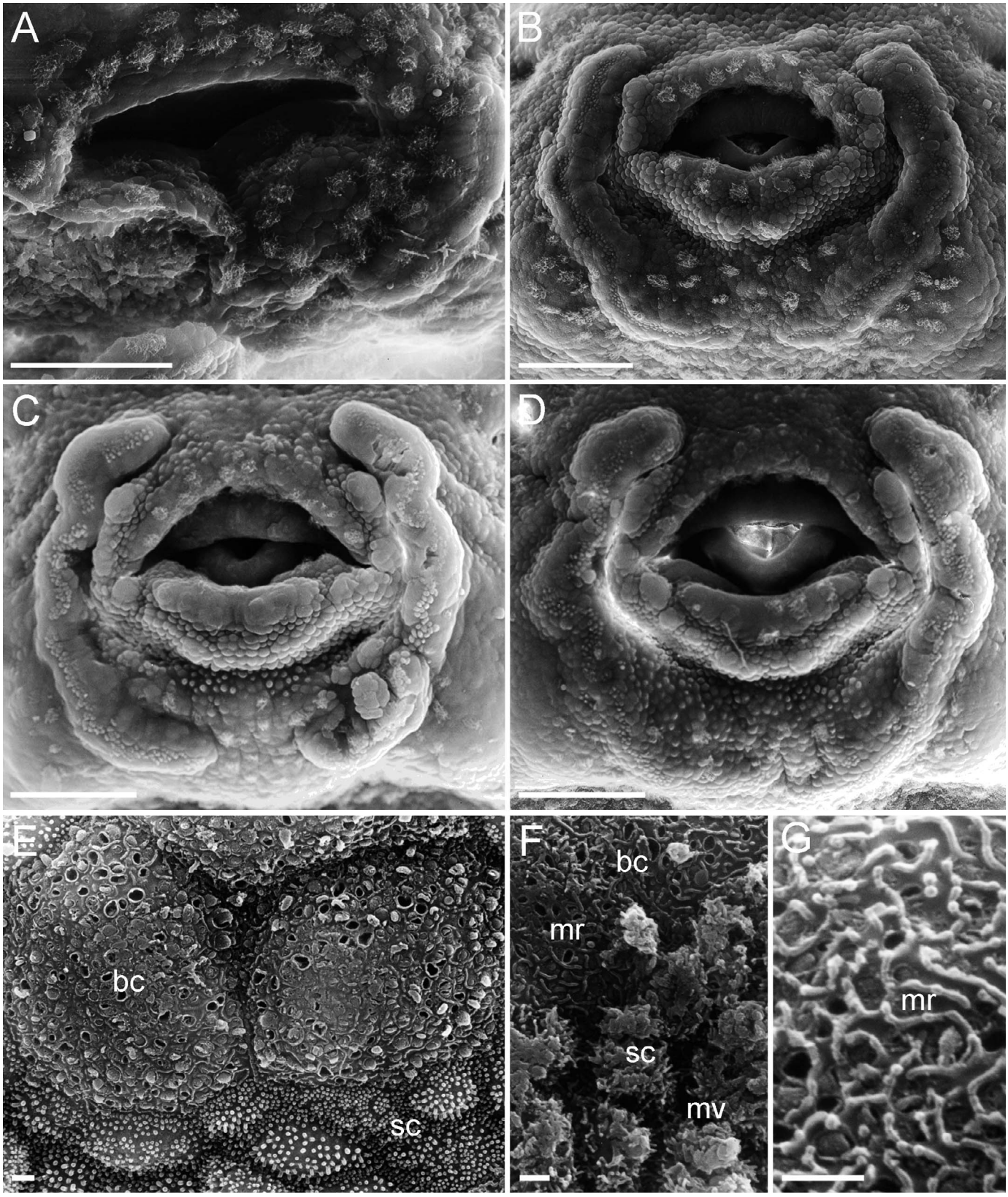

FIG. 3. Development of the adhesive glands of *Phyllomedusa azurea*. (A) K19/20. (B) K22. (C) K23. (D) K > 23. (E–G) Details of superficial cell types at K > 23. bc, bordering cells; mr, microridges; sc, secretory cells; mv, microvilli. Scale lines = 100 μm (A–D) and 1 μm (E–G).

occurs irregularly, resulting in the secretory region appearing discontinuous, with several remaining patches of secretory cells. The discontinuous pattern in *P. iheringii* then could be the result of an early regression of the gland. In *P. azurea*, regression occurs in a posterior to anterior direction such that the last region to lose cell differentiation is the one anterior to the oral disc (Figs. 3D and 6D); in the remaining species, the placement of the whole gland changes from posterolateral to anterolateral as the

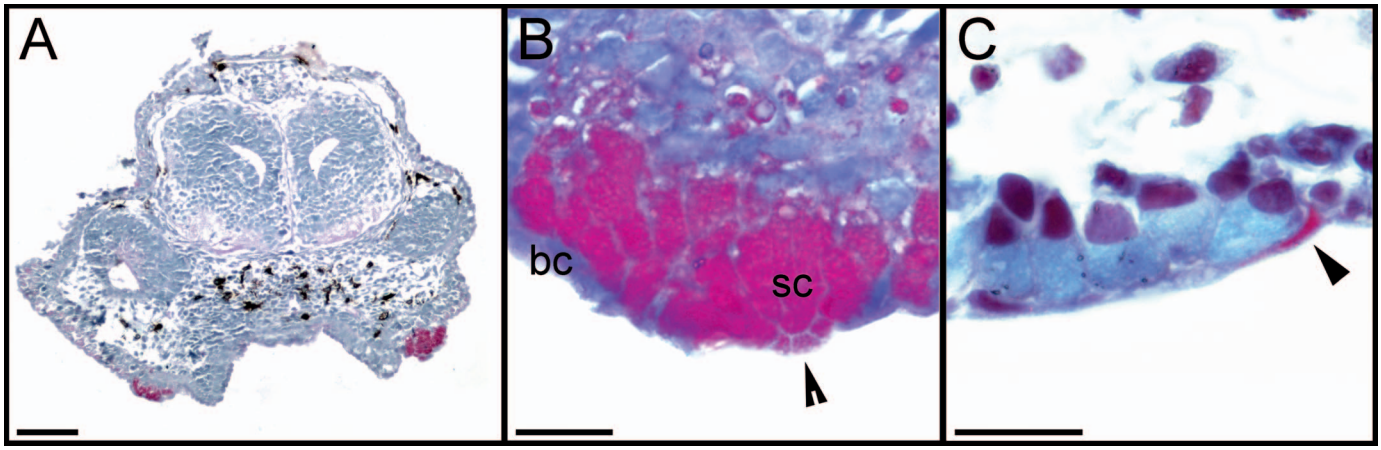

FIG. 4. Histological features of *Phyllomedusa azurea* glands at K23 (Alcian Blue-PAS). (A) Transverse, topographic view at the level of eyes. (B) details of secretory and bordering cells. Note the apical free extreme of PAS positive secretory cells (arrow tip). (C) Detail of an AB positive cell in an area adjacent to the adhesive gland (black arrow). Scale lines = 50 μm (A), 2 μm (B), and 20 μm (C).

regression progresses (compare Figs. 1 with 6A–C). We observed a similar regression pattern in *P. minuta* embryos (Hylidae, Dendropsophini; Fig. 6E), and this is different from how glands regress in other embryos with a C-morphogenetic pattern, where glands remain lateral or posterolateral to the oral disc until they disappear (e.g., *Dendropsophus* and *Scinax* in Fig. 6F,G; species with C-type in Nokhbatolfoghahai and Downie; FVC unpubl. data). Conversely, in *A. aspera* embryos, gland regression occurs synchronically across the whole gland (Fig. 6H).

The study of a relatively low number of species (only 10 of the 60 included in the subfamily; Frost, 2015) reveals an important level of diversity in the adhesive glands of Phyllomedusinae. Thus far, this diversity includes the absence of the adhesive gland (*Phasmahyla cochranae*, *Phyllomedusa boliviana*, and *Phyllomedusa trinitatis*); club-shaped Type-C glands (*Phyllomedusa sauvagii*, *Phyllomedusa tetraploidea*, *Phyllomedusa iheringii*, and *Phyllomedusa rohdei*); and extensive, oblong Type–C-like glands (*Phyllomedusa azurea* and *Agalychnis*). Variation also includes glands with dispersed secretory patches (*P. iheringii* and *A. aspera*) and glands with a single, central region of secretory cells (all remaining species).

We still lack information on gland occurrence and development in the basal genera of Phyllomedusinae (e.g., *Phrynomedusa*, *Cruziohyla*) and in the basal taxa of the two major clades of *Phyllomedusa* (*Phyllomedusa bicolor* and *Phyllomedusa vaillanti*, and the clade including *Phyllomedusa tomopterna* and the *Phyllomedusa perinesos* group). The little information we have today, however, optimized on the phylogenetic hypothesis of Faivovich et al. (2010) would imply an independent origin of the Type-C glands in *P. sauvagii* + the *Phyllomedusa burmeisteri* group and in at least *P. rohdei*. In the former clade, Type C glands evolved from a plesiomorphically absent state. In the latter case, however, the plesiomorphic state cannot be inferred, because *P. azurea* has the different, Type–C-like glands. The occurrence of both Type-C and Type–C-like morphogenetic patterns in *P. azurea* and *P. rohdei*, the only two studied exemplars of the *Phyllomedusa hypochondrialis* group, indicates that one might have evolved from the other, but this will remain ambiguous. Further study of the taxonomic distribution of the morphogenetic patterns in other species of the *P. hypochondrialis* group would allow an understanding of the evolution of the two patterns in the group. The Type–C-like glands independently occurring in *Agalychnis* have an ambiguous origin because they could be plesiomorphic for Phyllomedusinae, depending on the conditions present in *Cruziohyla* and *Phrynomedusa*. Furthermore, observations on gland occurrence and development in the sister taxon of phyllomedusines, the subfamily Pelodryadinae, are scarce, and illustrations and descriptions suggest either a Type-A (because of an initial crescentic organ) or Type-C (because of the early division of that structure) morphogenetic pattern (Anstis, 1976; Davies, 1989; Davies and Richards, 1990; Anstis and Tyler, 2005).

Knowledge on the diversity and taxonomic distribution of the adhesive glands in anurans is still quite poor; however, on the basis of the published information and unpublished data (FVC unpubl. data), the variation found in Phyllomedusinae is remarkable. Although the comparative study in other groups would help to better appreciate how common is this diversity, the functional consequences of morphological and developmental variation in adhesive glands of phyllomedusines should be particularly explored, including their relationship with hatching and their role in adherence to surfaces.

Hatching in embryos of phyllomedusines occurs late in comparison with other anurans, and most specimens reach the water at K23–24, with external gills still being well developed (Kenny, 1968; Pyburn, 1980; Lescure et al., 1995; Warkentin, 1999; Vargas and Gutiérrez, 2005). *Agalychnis lemur*, *Phasmahyla cruzi*, *Phasmahyla guttata*, and *Phyllomedusa trinitatis* are described to hatch at Gosner stage 24–25, i.e., with almost complete resorption of external gills (Jungfer and Weygoldt, 1994; Costa and Carvalho-e-Silva, 2008; Costa et al., 2010; Downie et al., 2013). In our studied species, embryos of *Phyllomedusa* hatch with well-developed gills, being the earliest hatchlings some of *P. azurea* at K23 (Table 1). Additionally, plastic hatching (i.e., the ability to hatch early in response to external stimuli) is known in species of the *A. callidryas* group, *Agalychonis dacnicolor*, *Cruziohyla calcarifer* (reviewed in Warkentin, 2007; Gomez-Mestre et al., 2008), whereas *P. trinitatis*, *P. sauvagii*, and *P. boliviana* embryos also hatch early when the eggs are immersed (Salica et al., 2011; Downie et al., 2013; MJS unpubl. data).

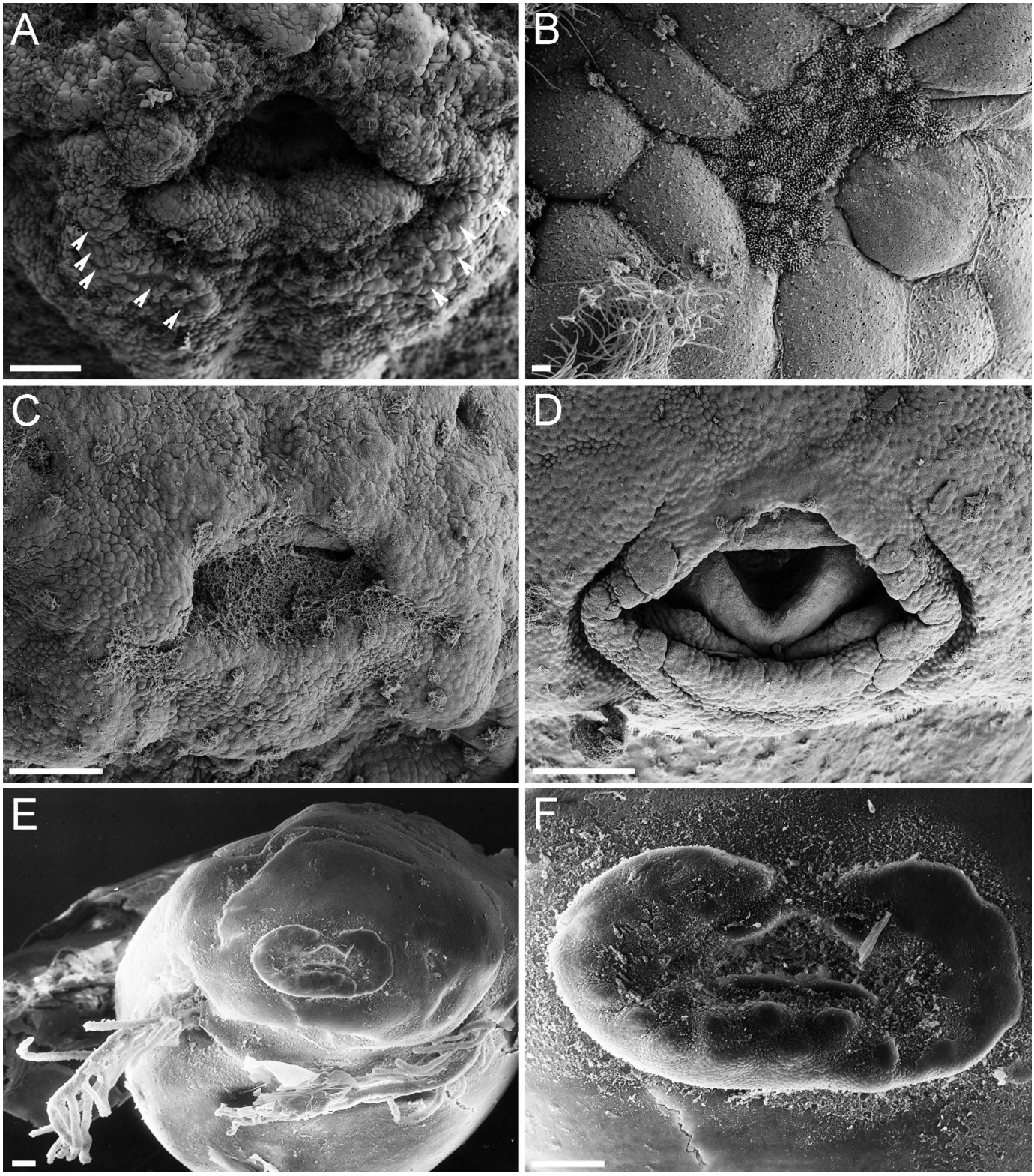

FIG. 5. Oral region in other phyllomedusines. (A, B) *Agalychnis aspera* K22, distribution of secretory patches (arrow tips) lateral and posterolateral to the oral disc, and detail of secretory cells. (C, D) *Phyllomedusa boliviana* K21/22 and K23. (E, F) *Phasmahyla cochranae* K22. Note the absent glands in these two latter species. Scale lines = 2 μm (B) and 100 μm in the others.

Several structures of the oral region likely are involved in stimuli detection to hatch and in the mechanism of hatching, including neuromasts, hatching gland cells, and the oral disc itself (Fig. 7). The oral lateral lines are evident at early, prehatching stages in most studied species, with developed neuromasts. In turn, cells of the hatching gland are typically distributed all over the rostral region in possibly all phyllomedusines (Downie et al., 2013; we have even seen hatching cells scattered among groups of secretory cells of the adhesive glands in *A. aspera*, Fig. 7D,E), very different from the dorsal

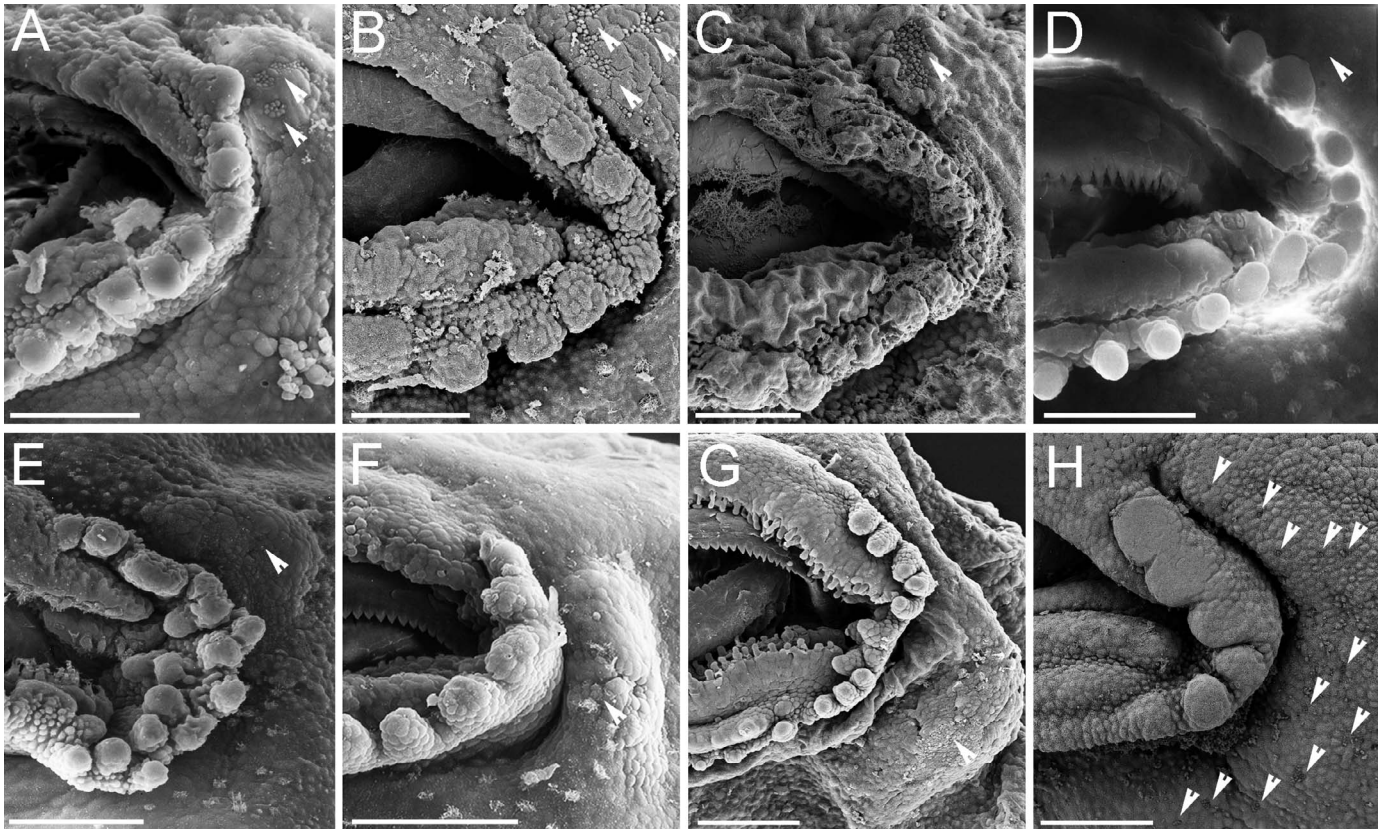

FIG. 6. Regression patterns of glands in phyllomedusine and dendropsophini hylids. Regressing glands are evident as patches of secretory cells (arrow tips) anterolateral to the oral disc in: (A) *Phyllomedusa sauvagii* K24. (B) *Phyllomedusa iheringii* K24. (C) *Phyllomedusa tetraploidea* K24. (D) *Phyllomedusa azurea* K24. (E) *Pseudis minuta* Gosner Stage >25. Regressing glands at the commissures of the oral disc: (F) *Dendropsophus minutus* Gosner Stage >25. Regressing glands posterolateral to the oral disc: (G) *Scinax uruguayus* Gosner Stage 25. Regressing glands anterolateral, lateral and posterior to the oral disc: (H) *Agalycnhis aspera* K24. Scale lines = 100 μm.

arrangement that occurs in embryos from other studied anurans (Nokhbatolfoghahai and Downie, 2007). A possible functional role of adhesive glands during hatching, like that suggested by Bles (1905) for *X. laevis*, should be further investigated in phyllomedusines. High speed videos of *Agalychnis callidryas* show that the embryos contact the vitelline membrane for a few seconds before hatching (Cohen et al., 2016). Whether adhesive glands are involved in this behavior remains unknown, and also, detailed observations of hatching embryos without adhesive glands are needed to compare hatching mechanisms and performances. Previous studies relate the absence of adhesive glands with a late hatching (Nokhbatolfoghahai and Downie, 2005; Nokhbatolfoghahai et al., 2015); however, observations in Phyllomedusinae show that this may not be the case, because embryos hatch late independently of the absence (e.g., *P. boliviana*) or presence (e.g., *P. azurea*) of adhesive glands. Furthermore, plastic hatching is independent from gland presence, because variations in the time of hatching are observed in embryos with (e.g., *P. sauvagii*) and without (e.g., *P. trinitatis*) adhesive glands.

After hatching, phyllomedusine embryos usually adhere to the surface film of the water, hanging from their oral discs (e.g., Kenny, 1968; Warkentin, 1999). Prematurely hatched embryos of *A. callidryas* do not succeed in this behavior, but they adhere to surfaces within the water through their adhesive glands (Warkentin, 1999). In *X. laevis* embryos, Lambert et al. (2004) demonstrated that, while attached, swimming activity and responsiveness to swim-initiating stimuli are reduced over long periods of time; embryos that do not move are then less likely to be detected, and predation risk decreases. Given these observations, some compensatory mechanism (e.g., an early development of buoyancy or locomotion) could be suspected to occur in those early hatched embryos without adhesive glands. Detailed in vivo observations of hatching embryos, along with histochemical studies of hatching and adhesive gland secretions are needed to understand the complex behaviors and mechanisms of hatching and surface adherence in anuran embryos.

Acknowledgments. This work was supported by Agencia Nacional de Promoción Científica y Tecnológica (PICT 1524/2011, 1895/2011, 2687/2012, 0404/2013, 1343/2014 and 1930/2014), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 0875 and 0889), Universidad Nacional de Tucumán (PIUNT G519), and Grants 2012/10000-5, 2012/25370-2 and 2013/50741-7, São Paulo Research Foundation (FAPESP). We thank Unidad Ejecutora Lillo (CONICET–Fundación Miguel Lillo), Centro Integral de Microscopía Electrónica (CIME, Tucumán), and Laboratorio de Microscopía Electrónica de Barrido de la Universidad Nacional de Salta (LASEM, Salta), and Reserva Particular do Patrimônio Natural Estação Veracel (RPPN Veracel), and Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio-IBAMA, license numbers 41508-5) for issued collecting permits. We are indebted to F. Kolenc and C.

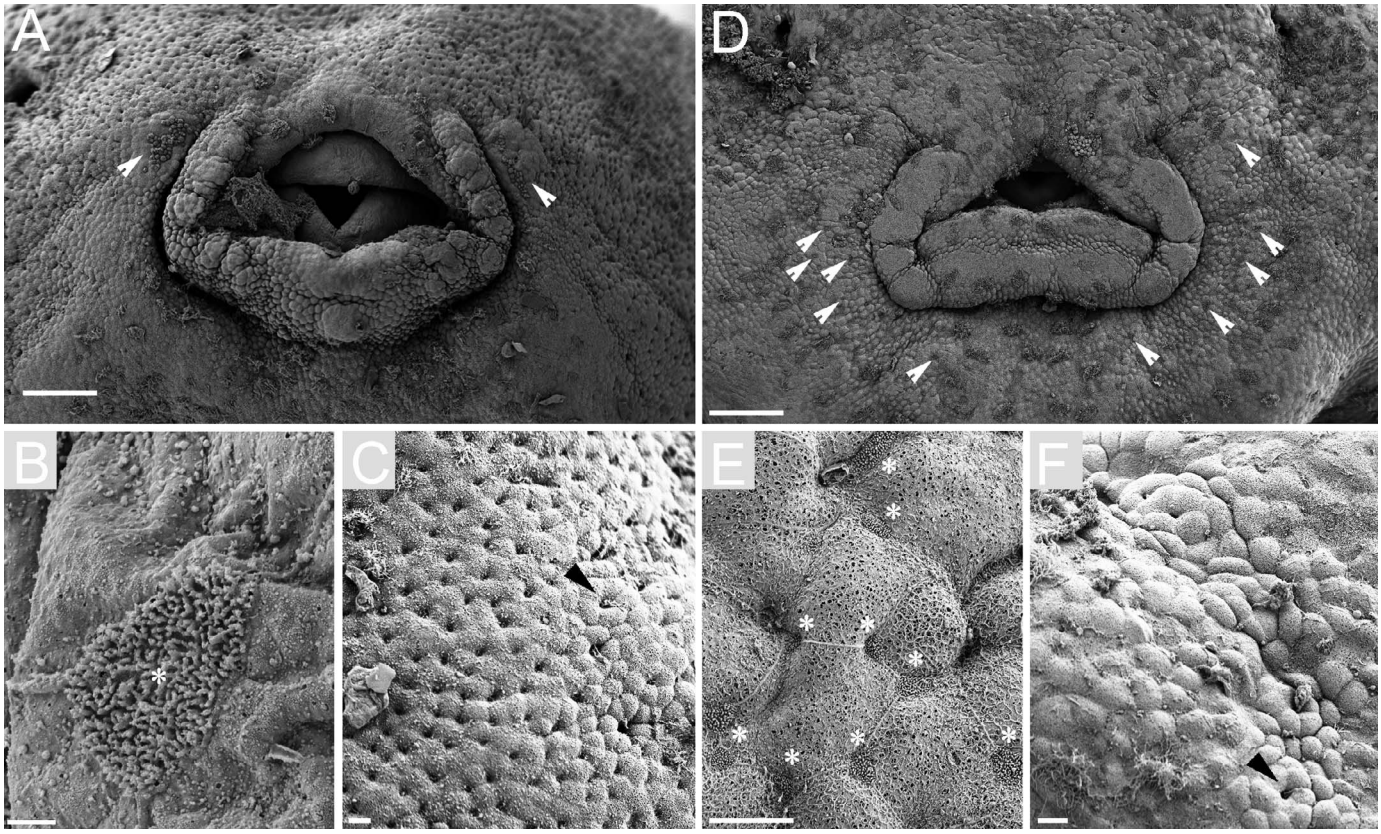

FIG. 7. Oral region in pre-hatching embryos of *Phyllomedusa tetraploidea* (A–C) and *Agalychnis aspera* (D–F), showing topographic distribution of secretory patches of adhesive glands (white arrow tips), details of hatching gland cells between the oral disc and nares (asterisks in B and E), and oral neuromasts (black arrows in C and F). Scale lines = 100 μm (A,D), 10 μm (C,E,F), and 1μm (B).

Borteiro for preparing *P. iheringii* series and to M. Akmentins for collecting *P. boliviana* nests.

LITERATURE CITED

ALTIG, R. 2007. A primer for the morphology of anuran tadpoles. Herpetological Conservation and Biology 2:71–74.

ALTIG, R,, AND R. W. MCDIARMID. 1999. Diversity: familial and generic characterization. Pp. 295–337 in R. W. McDiarmid and R. Altig (eds.), Tadpoles: The Biology of Anuran Larvae. University of Chicago Press, USA.

ANSTIS, M. 1976. Breeding biology and larval development of *Litoria verreauxi* (Anura: Hylidae). Transactions of the Royal Society of South Australia 100:193–202.

ANSTIS, M., AND M. J. TYLER. 2005. Breeding biology of *Litoria microbelos* (Cogger) (Anura: Hylidae). Transactions of the Royal Society of South Australia 129:43–48.

BLES, E. J. 1905. The life history of *Xenopus laevis* Daudin. Transactions of the Royal Society of Edinburgh 41:789–821.

BUDGETT, J. S. 1899. Notes on the batrachians of the Paraguayan Chaco, with observations upon their breeding habits and development, especially with regard to *Phyllomedusa hypochondrialis*, Cope. Also a description of a new genus. Quarterly Journal of Microscopical Science 42:305–333.

COHEN, K. L., M. SEID, AND K. M. WARKENTIN. 2016. How embryos escape from danger: the mechanism of rapid, plastic hatching in red-eyed treefrogs. Journal of Experimental Biology 219:1875–1883.

COSTA, P. N., AND A. M. T. CARVALHO-E-SILVA. 2008. Ontogenia e aspectos comportamentais da larva de *Phasmahyla guttata* (Lutz, 1924) (Amphibia, Anura, Hylidae). Biota Neotropica 8:219–224.

COSTA, P. N., A. M. T. CARVALHO-E-SILVA, AND A. FLASKMAN. 2010. Egg clutch and larval development of *Phasmahyla cruzi* Carvalho-e-Silva, Silva and Carvalho-e-Silva, 2009 (Amphibia, Anura, Hylidae). Herpetology Notes 3:221–228.

DAVIES, M. 1989. Developmental biology of the australopapuan hylid frog *Litoria eucnemis* (Anura: Hylidae). Transactions of the Royal Society of South Australia 113:215–220.

DAVIES, M., AND S. J. RICHARDS. 1990. Developmental biology of the Australian hylid frog *Nyctimystes dayi* (Günther). Transactions of the Royal Society of South Australia 114:207–211.

DE SÁ, R. AND A. GERHAU. 1983. Observaciones sobre la biología de *Phyllomedusa iheringii* Boulenger 1885 (Anura, Hylidae). Boletín de la Sociedad Zoológical del Uruguay 1:44–49.

DIAS, T. M., F. P., MARAGNO, B. MADALOZZ, C. PRADO, AND S. Z. CECHIN. 2013. Breeding sites of the leaf frog *Phyllomedusa tetraploidea* (Hylidae, Phyllomedusinae) in a forest remnant in southern Brazil. North-Western Journal of Zoology 9:422–424.

DOWNIE, J. R, M. NOKHBATOLFOGHAHAI, D. BRUCE, J. M. SMITH, N. ORTHMANN-BRASK, AND I. MACDONALD-ALLAN. 2013. Nest structure, incubation and hatching in the Trinidadian leaf-frog *Phyllomedusa trinitatis* (Anura: Hylidae). Phyllomedusa 12:13–32.

FAIVOVICH, F., C. F. B. HADDAD, D. BAÊTA, K. H. JUNGFER, G. F. R. ALVARES, R. A. BRANDÃO, C. SHEIL, L. S. BARRIENTOS, C. L. BARRIO-AMORÓS, C. A. G. CRUZ ET AL. 2010. The phylogenetic relationships of the charismatic poster frogs, Phyllomedusinae (Anura, Hylidae). Cladistics 25:1–35.

FROST, D. R. 2015. Amphibian Species of the World: An Online Reference. Version 6.0. Available from: http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York.

GOMEZ-MESTRE, I., J. J. WIENS, AND K. M. WARKENTIN. 2008. Evolution of adaptive plasticity: risk-sensitive hatching in Neotropical leaf-breeding treefrogs. Ecological Monographs 78:205–224.

GOSNER, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16:183–190.

JUNGFER, K. H., AND P. WEYGOLDT. 1994. The reproductive biology of the leaf frog *Phyllomedusa lemur*, Boulenger 1882, and a comparison with other members of the Phyllomedusinae (Anura: Hylidae). Revue Francaise d'Aquariologie et Herpetologie 21:57–64.

KENNY, J. S. 1968. Early development and larval natural history of *Phyllomedusa trinitatis* Mertens. Caribbean Journal of Science 8:35–45.

Lambert, T. D., J. Howard, A. Plant, S. Soffe, and A. Roberts. 2004. Mechanisms and significance of reduced activity and responsiveness in resting frog tadpoles. Journal of Experimental Biology 207:1113–1125.

Lescure, J., C. Marty, V. Marty, F. Starace, M. Auber-Thomay, and F. Letellier. 1995. Contribution a l'étude des amphibiens de Guyane Francaise. X. Les *Phyllomedusa* (Anura, Hylidae). Revue Francaise d'Aquariologie et Herpetologie 22:35–50.

Lutz, A., and B. Lutz. 1939. I. Notes on the genus *Phyllomedusa* Wagler. A) Observations on small Phyllomedusae without vomerine teeth or conspicuous parotids found in the region of Rio de Janeiro. B) *Phyllomedusa bahiana* Lutz. Anais da Academia Brasileira de Ciências 11:219–263.

Mowry, R. W. 1956. Observations on the use of sulphuric ether for the sulphation of hydroxyl groups in tissue sections. Journal of Histochemistry and Cytochemistry 4:407.

Nokhbatolfoghahai, M., and J. R. Downie. 2005. Larval cement gland of frogs: comparative development and morphology. Journal of Morphology 263:270–283.

———. 2007. Amphibian hatching gland cells: pattern and distribution in anurans. Tissue and Cell 39:225–240.

———. 2008. The external gills of anuran amphibians: comparative morphology and ultrastructure. Journal of Morphology 269:1197–1213.

Nokhbatolfoghahai, M., J. R. Downie, A. K. Clelland, and K. Rennison. 2005. The surface ciliation of anuran amphibian embryos and early larvae: patterns, timing differences and functions. Journal of Natural History 39:887–929.

Nokhbatolfoghahai, M., J. R. Downie, and V. Ogilvy. 2006. Surface ciliation of anuran amphibian larvae: persistence to late stages in some species but not others. Journal of Morphology 267:1248–1256.

Nokhbatolfoghahai, M., C. J. Pollock, and R. Downie. 2015. Oviposition and development in the glass frog *Hyalinobatrachium orientale* (Anura: Centrolenidae). Phyllomedusa 14:3–17.

Pennati, R., A. M. Bolzern, S. Groppelli, C. Sotgia, and F. D. Bernardi. 2000. The adhesive organs of Anura: a histological and molecular study. Italian Journal of Zoology 67:1–8.

Perotti, M. G. 1995. Biología y reproducción de anuros chaqueños. Unpubl. thesis. Univ. Nac. Tucumán, Argentina.

Picard, J. J. 1976. Ultrastructure of the cement gland of *Xenopus laevis*. Journal of Morphology 148:193–208.

Pimenta, B. V. S., I. Nunes, and C. A. G. Cruz. 2007. Notes on the poorly known Phyllomedusine frog *Hylomantis aspera,* Peters 1872 (Anura, Hylidae). South American Journal of Herpetology 2:206–214.

Pyburn, W. F. 1963. Observations on the life history of the treefrog *Phyllomedusa callidryas* (Cope). Texas Journal of Science 15:155–170.

———. 1980. The function of the eggless capsules and leaf in nests of the frog *Phyllomedusa hypochondrialis* (Anura: Hylidae). Proceedings of the Biological Society of Washington 93:153–167.

Rodrigues, D., M. Uetanabaro, and F. Lopes. 2007. Breeding biology of *Phyllomedusa azurea* Cope, 1862 and *P. sauvagii,* Boulenger 1882 (Anura) from the Cerrado, Central Brazil. Journal of Natural History 41:1841–1851.

Sacramento, M., D. B. Provete, and V. X. da Silva. 2013. Descrição da biologia reprodutiva de *Phasmahyla cochranae,* Bokermann 1966 (Anura, Hylidae). 3rd Congresso Brasileiro de Herpetologia 2007, Vol. 4.

Salica, M. J., M. B. Haad, F. Vera Candioti, and J. Faivovich. 2011. Early development of two species of *Phyllomedusa* (Anura: Phyllomedusinae). Salamandra 47:144–154.

Vaira, M. 2001. Breeding biology of the leaf frog, *Phyllomedusa boliviana* (Anura, Hylidae). Amphibia–Reptilia 22:421–429.

Vargas, S., and C. Gutiérrez. 2005. Cambios morfológicos y mortalidad en embriones y renacuajos de *Agalychnis spurrelli* Boulenger (Anura: Hylidae). Actualidades Biológicas 27:189–202.

Wardle, F. C., and H. L. Sive. 2003. What's your position? The *Xenopus* cement gland as a paradigm of regional specification. Bioessays 25: 717–726.

Warkentin, K. 1999. Effects of hatching age on development and hatchling morphology in the red-eyed tree frog, *Agalychnis callidryas*. Biological Journal of the Linnean Society 68:443–470.

———. 2007. Oxygen, gills, and embryo behavior: mechanisms of adaptive plasticity in hatching. Comparative Biochemistry and Physiology A 148:720–731.

Accepted: 15 August 2016.
Published online: 12 January 2017.

Appendix 1

Dendropsophini hylid embryos examined for comparison.

Dendropsophus sanborni. Uruguay: Artigas: Bella Unión, Rincón de Franquía, LGE 10204. Uruguay: Montevideo: Salida MNHN a San José, LGE 10207.

Dendropsophus minutus. Uruguay: Treinta y Tres: Camino a Isla Patrulla por detrás de la quebrada, LGE 10208. Argentina: Misiones, Leandro Alem, Colonia Alemana, LGE 05075.

Pseudis minuta. Uruguay: Treinta y Tres: Valentines, LGE 10199. Uruguay: Rocha: Laguna de Rocha, LGE 10205.

Scinax fuscovarius. Argentina: Tucumán: San Javier, Laguna Ciudad Universitaria, LGE 00139. Argentina: Misiones: Mojón Grande, Arroyo Guerrero, Ruta Provincial No. 209, LGE 07325.

Scinax uruguayus. Uruguay: Maldonado: Ruta 109 límite con Rocha, LGE 10469.