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Structural and Heterochronic Variations During the Early Ontogeny in Toads (Anura: Bufonidae)

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Abstract: In recent decades, a renewed interest in comparative studies of embryonic ontogeny in anurans is taking place. Toad embryos are often employed as model organisms, and scarce attention has been put on interspecific variations. In this work we analyze the development of transient embryonic and larval structures in 21 species in five genera of Bufonidae. These species vary in their ovipositional mode and the type of environments where the embryos and tadpoles develop, including ponds, streams, and axils of leaves of terrestrial or epiphytic plants. Comparative anatomical studies and sequence heterochrony analyses show that primary morphological variations occur in the morphology at the tail-bud stage, the arrangement and development of the external gills, adhesive gland type and division timing, growth of the dorsal hatching gland on the head, configuration of the oral disc, emergence and development of the hind limbs, and presence of the abdominal sucker. Some of these transformations are best explained by phylogeny (e.g., early divergent taxa of bufonids have embryos with kyphotic body curvature, Type C adhesive glands, and a very small third pair of gills). Other traits might be correlated with reproductive modes (e.g., phytotelmata embryos hatch comparatively late and show an accelerated development of hind limbs). Because these actual variations are not well studied (e.g., less than the 10% of the known diversity of bufonids has been studied from this perspective), comprehensive analyses are required to interpret character evolution and the relationship with reproductive modes within the family.

Key words: Adhesive glands; *Atelopus*; *Bufotes*; *Dendrophryniscus*; External gills; Hatching glands; *Melanophryniscus*; Oral disc; *Rhinella*

Comparative research on embryonic development in anurans is undergoing a period of renewed interest, comparable to that present in earlier days of developmental biology. Landmark studies by Nokhbatolfoghahai and collaborators (Nokhbatolfoghahai and Downie 2005, 2007, 2008; Nokhbatolfoghahai et al. 2005, 2006) have shown that morphology during embryogenesis, with its exclusive transient structures that regress after hatching (i.e., hatching and adhesive glands, external gills, and ciliated cells), is at least as diverse as it is during larval and later stages of anuran ontogeny. In turn, typically larval features (e.g., the oral disc) begin to develop at these early stages, and much of the phenotypic variation in tadpoles might be a consequence of changes in their developmental pathways (e.g., Vera Candioti et al. 2011). All these variations can be related to both historical and ecological aspects, and general patterns previously described serve as a basis for further inquires.

Embryos of bufonids are often employed as model organisms (e.g., *Bufo bufo*, *Rhinella arenarum*, and *R. marina*; e.g., Aceto et al. 1993; Weber et al. 1994; Lascano et al. 2009), and little attention has been focused on interspecific variations. In this work we analyzed 21 species in five genera of the family Bufonidae. Sampling was concentrated on the neotropical genera *Melanophryniscus* and *Rhinella*. *Melanophryniscus* is particularly interesting because of its early divergence within bufonids, whereas *Rhinella* is one of the most diverse and widely distributed genera of this family (Frost et al. 2006; Pereyra et al. 2016). We also included species of the genera *Atelopus* and *Dendrophryniscus* that also diverged early within the family. *Atelopus* tadpoles have an abdominal sucker that allows larvae to attach to substrates in lotic systems (e.g., Rueda-Solano et al. 2015). *Dendrophryniscus* larvae develop in small terrestrial pools and in tanks of bromeliads (Izecksohn and da Cruz 1972). Finally, we included observations on *Bufotes viridis* to extend our comparisons to a derived and fairly well-known Old-World bufonid taxon (e.g., Bonacci et al. 2008). The analyses compare the development of the four transient structures studied by Nokhbatolfoghahai and collaborators: the hatching gland, adhesive glands, external gills, and ciliated cells, plus the larval oral disc. We paid specific attention to changes in the sequence in which developmental events occur (sequence heterochrony; Smith 2001). As mentioned by Chipman et al. (2000), knowledge of ontogenetic diversity is a requirement to understand the evolution of the development in anurans and changes in patterns and mechanisms across lineages.

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Materials and Methods

We studied ontogenetic series of 21 bufonid species. Eight species of *Melanophryniscus* include two basal species for the genus, *M. krauczuki* and *M. sanmartini*, and representatives of the three intrageneric groups (D. Baldo, personal observation): *M. atroluteus* and *M. rubriventris* (*M. stelzneri* group); *M. alipioi* and *M. milanoi* (*M. moreirae* group); and *M.* aff. *devincenzii* and *M. macrogranulosus* (*M. tumifrons* group). Nine species of *Rhinella* belong to five of seven intrageneric groups: *R. ornata* (*R. crucifer* group); *R. azarai*, *R. fernandezae*, and *R. major* (*R. granulosa* group); *R. arenarum* and *R.* cf. *cerradensis* (*R. marina* group); *R. achalensis* and *R. spinulosa* (*R. spinulosa* group); and *R. rumbolli* (*R. veraguensis* group). The four remaining species are *Atelopus elegans* and *A.* aff. *spumarius*, *Bufotes viridis*, and *Dendrophryniscus* aff. *berthalutzae*. We collected clutches of each species in the field or from breeding populations in captivity (Appendix). The ovipositional modes include independent eggs in *M. alipioi* and *M. milanoi*, egg masses in all the remaining *Melanophryniscus*, egg clumps in *Dendrophryniscus* and *R. rumbolli*, and long strings of eggs in the remaining species (de Carvalho 1949; McDiarmid 1971; Bustos Singer and Gutiérrez 1997; Baldo and Basso 2004; Altig and McDiarmid 2007; Langone et al. 2008; Bornschein et al. 2015; Pereyra et al. 2015). Embryos vary widely in the type of environments where they develop, including ponds, streams, and axils of leaves of terrestrial or epiphytic plants. We raised clutches in containers with dechlorinated water; a portion of eggs was immediately fixed in 10% formalin, and then embryos were fixed every 6–8 h for about a week. The raising of embryos was usually performed in situ during the field trips, so we expect that the environmental variables did not change enough as to interfere with normal development. From the collected material, we obtained ontogenetic series by selecting embryos at successive developmental stages whenever a relevant morphological change was noticed. We mainly considered Gosner (1960) stages from 18 to 25, but other stages were included when necessary.

We examined and photographed specimens with a Leica M205 stereomicroscope; methylene blue was used to stain translucent structures such as papillae, labial ridges, and adhesive glands (Wassersug 1976). We also prepared some specimens (4–15 per species) for scanning electron microscopy (SEM) with the protocol of Fiorito de López and Echeverría (1984). For the characterization of embryonic structures, we followed Nokhbatolfoghahai and Downie (2005, 2007, 2008) and Nokhbatolfoghahai et al. (2006); the oral disc development followed the terminology of Thibaudeau and Altig (1988) and Vera Candioti et al. (2011).

We used the concept of sequence heterochrony (Smith 2001) to analyze the temporal shifts during the early development. This approach describes the ontogenetic trajectories as sequences of specific events that depict the development progression of different structures for each species and interprets the shifts of the event position as a heterochronic change. The use of absolute or relative time for comparative analyses can be particularly complicated in frog embryos because of the high developmental plasticity in response to external factors (Harkey and Semlitsch 1988; Love 2010). Thus, in this context the approach of stage-free, time-free sequence heterochrony emerges as a proper method to describe and compare ontogenetic trajectories in anuran early development. Although environmental conditions may alter the development periods, the development sequences may remain unmodified (Thibaudeau and Altig 1999). We defined 26 key events occurring in all species as follows: tail, (1) tail bud, (2) fin buds, (3) tail length/body length = 1; adhesive glands, (4) adhesive glands as a groove, (5) adhesive glands separated, (6) adhesive glands not visible; external gills, (7) branchial arches, (8) first gill pair bud, (9) first gill pair branched, (10) second gill pair bud, (11) second gill pair branched, (12) gill base covered by the operculum, (13) gills fully developed, considering the number and length of filaments, (14) operculum medially fused, (15) right gill covered by the operculum, (16) left gill covered by the operculum, (17) spiracle developed; oral disc, gut, and feeding, (18) labia outlined, upper labium curved and lower labium convex, (19) labial tooth row formula (LTRF) outlined, (20) first labial teeth, (21) marginal papilla full development (submarginal papillae not considered), (22) larval oral disc defined, (23) first gut coil, (24) active feeding (as inferred from food particles in the guts); and hind limbs, (25) hind limb buds, and (26) hind limb at Stage 27. The sequence of occurrence of events for each species was converted into a rank ordering. The earliest event was given the number 1 and the latest the number 26 (the total number of event in the series); the events that occurred synchronously were given the mean rank of all the events occurring at that time. The sequences for each species were verified by examining random embryos from the same and different ovipositions; unfortunately in most cases we could only collect a single oviposition, so the intraspecific variations emphasizes intraclutch effects. The sequences for all species were then compared in a two-axis graph, where events ordered as they occur in a reference trajectory were plotted against their rank number. As reference trajectory we used a developmental sequence of the hylid *Hypsiboas riojanus*.

Finally, we performed ancestral state reconstructions for the evolution of morphological and developmental characters on the phylogenetic hypothesis of Pyron (2014), to which we added species from the *Rhinella granulosa* and *R. marina* groups according to the phylogenetic analyses of Pereyra et al. (2016) and Maciel et al. (2010; also see Pramuk et al. 2008; van Bocxlaer et al. 2010; Ron et al. 2015). Most parsimonious ancestral state reconstructions were performed with Mesquite v3.03 (Maddison and Maddison 2015) by considering all characters as unordered (Fitch 1971). Specimens are housed in the herpetological collections detailed in the Appendix; we use the museum acronyms of Sabaj (2016).

Results and Discussion

The early developmental patterns of *Melanophryniscus* aff. *devincenzii* and *Rhinella* cf. *cerradensis* are depicted to summarize the primary differences between the two genera (Fig. 1). Figures 2–25 illustrate structural changes in embryonic and larval features of the other bufonids that we studied. Sequences of developmental events are listed for all species in Table 1, and Fig. 26 shows the sequence heterochrony plots for the bufonids relative to a hylid



Fig. 1.—Developmental series of *Melanophryniscus* aff. *devincenzii* (left) and *Rhinella* cf. *cerradensis* (right) from tail-bud stage to spiracle differentiation. Note main differences in embryo pigmentation, size, and shape. Scale lines = 1 mm. A color version of this figure is available on line.

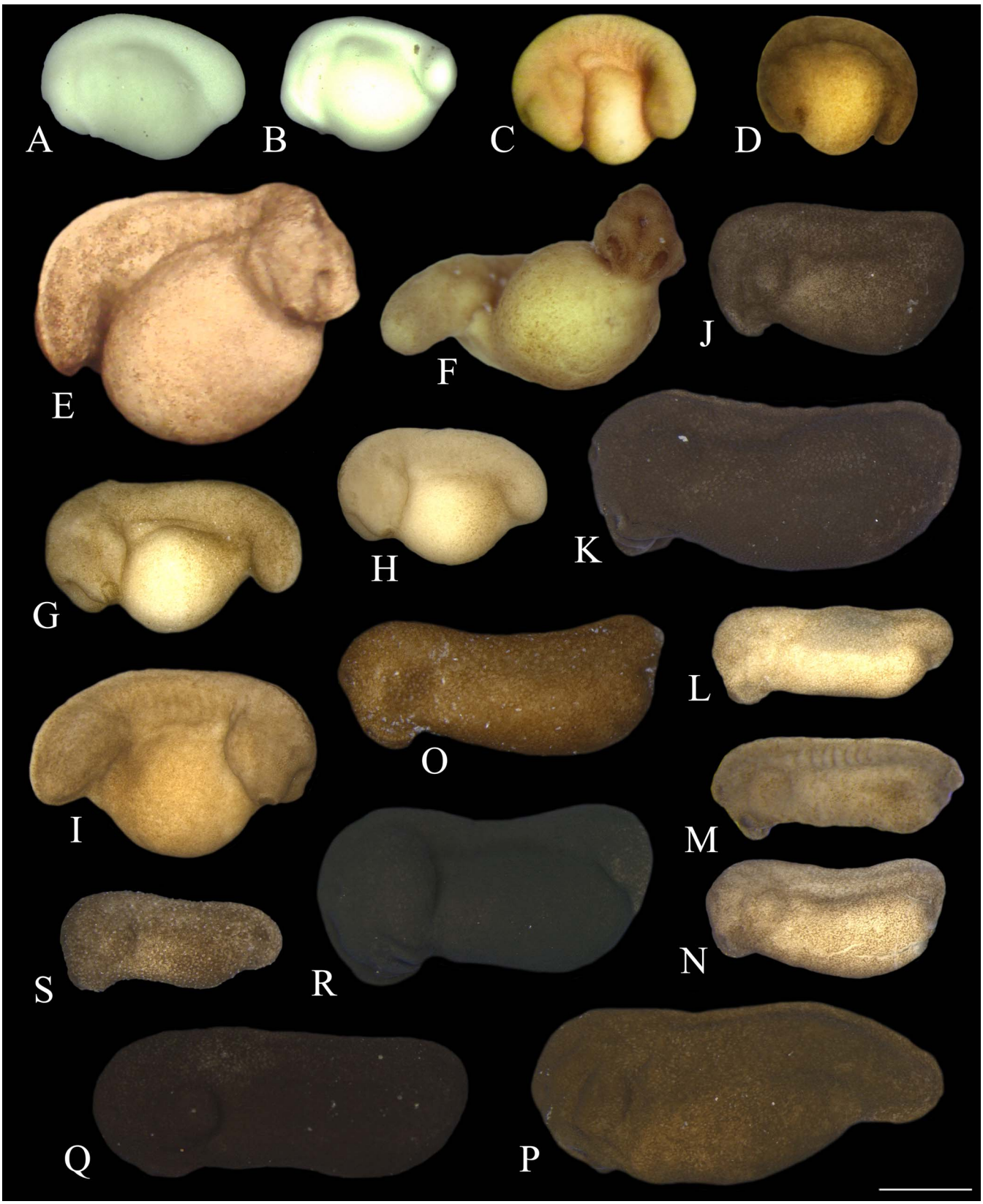


FIG. 2.—Differences in size, shape, and pigmentation of embryos at the tail-bud stage in Bufonidae. (A) *Atelopus elegans*, (B) *A.* aff. *spumarius*, (C) *Melanophryniscus krauczuki*, (D) *M. sanmartini*, (E) *M. alipioi*, (F) *M. macrogranulosus*, (G) *M.* aff. *devincenzii*, (H) *M. atroluteus*, (I) *M. rubriventris*, (J) *Rhinella arenarum*, (K) *R.* cf. *cerradensis*, (L) *R. fernandezae*, (M) *R. major*, (N) *R. azarai*, (O) *R. ornata*, (P) *R. rumbolli*, (Q) *R. achalensis*, (R) *R. spinulosa*, (S) *Bufotes viridis*. *Melanophryniscus milanoi* and *Dendrophryniscus* aff. *berthalutzae* are not included because we lack early embryos at this stage. Scale line = 1 mm. A color version of this figure is available on line.

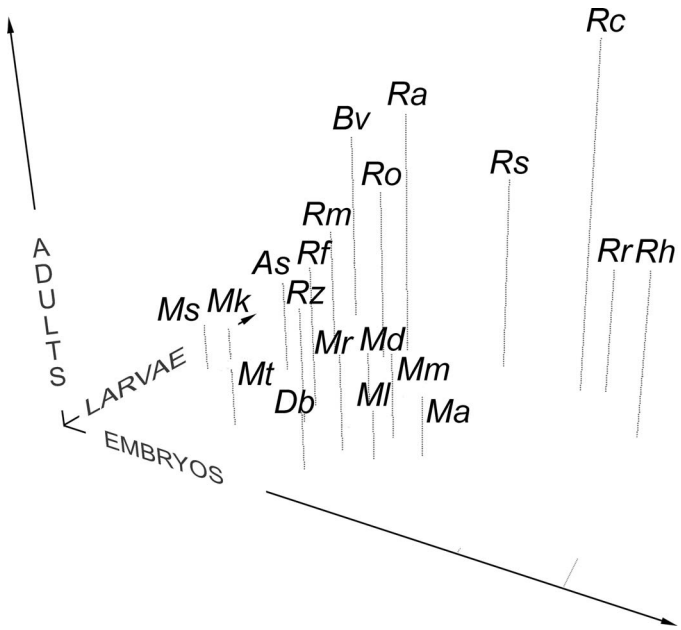


Fig. 3.—A scatterplot of bufonid species in a three-dimensional space defined by embryo, tadpole, and adult sizes (values from Appendix). As, *A.* aff. *spumarius*; Bv, *Bufotes viridis*; Db, *Dendrophryniscus* aff. *berthalutzae*; Ma, *Melanophryniscus alipioi*; Md, *M.* aff. *devincenzii*; Mk, *M. krauczuki*; Ml, *M. milanoi*; Mm, *M. macrogranulosus*; Mr, *M. rubriventris*; Ms, *M. sanmartini*; Mt, *M. atroluteus*; Ra, *Rhinella arenarum*; Rc, *R.* cf. *cerradensis*; Rf, *R. fernandezae*; Rh, *R. achalensis*; Rm, *R. major*; Ro, *R. ornata*; Rr, *R. rumbolli*; Rs, *Rhinella spinulosa*; Rz, *R. azarai*. Note the small stages of *M. atroluteus*, the large stages of *R.* cf. *cerradensis*, and the large embryos combined with minute adults in *M. alipioi*.

trajectory (*Hypsiboas riojanus*). Morphological and heterochronic variations are summarized in the sections below.

Color, Size, and Shape (Figs. 2, and 3)

Our observations and previous data show that the pigmentation at tail-bud stage (Stage 17) ranges from completely unpigmented (e.g., *Atelopus*; Fig. 2A,B; Gawor et al. 2012) to densely pigmented embryos that develop from pigmented eggs (e.g., *Rhinella* cf. *cerradensis* and *R. spinulosa* in Fig. 2K,R; Pereyra et al. 2015). Embryos of the *R. granulosa* group have a paler pigmentation similar to species of *Melanophryniscus* (Fig. 2L–N and C–I; Pereyra et al. 2015). Pigmentation changes during ontogeny also vary. In *Atelopus* species, chromatophores appear on the lateral regions and the base of the tail only when the gill buds develop. All the other species are pigmented by early stages, and pigmentation increases with development. Dark pigmentation of eggs may protect against solar UV radiation or capture solar heat required to accelerate embryo development; conversely, eggs that develop in concealed, shady places are often less pigmented (e.g., Duellman and Trueb 1986; Elinson and del Pino 2012). Accordingly, Salazar-Nicholls and del Pino (2015) found coloration differences between eggs of two centrolenid species that develop at the underside or the upper surface of plant leaves. They also suggested that differences in the color of hatchlings might be related to occurrence in dissimilar aquatic environments. Several *Atelopus* lay their unpigmented eggs either on top or below submerged surfaces (e.g., Karraker et al. 2006), and the Poole (2006) comments about light sensitivity of *A. zeteki* eggs should be further investigated in other species. Within *Rhinella* and *Melanophryniscus*, differences in oviposition sites are not sufficient to explain differences in eggs and embryo coloration. Eggs and embryos are plesiomorphically pigmented (although at different degree) in Bufonidae, but at least three independent transformations occur in *Atelopus* and the viviparous species *Nimbaphrynoides occidentalis* (matrotrophic) and *Nectophrynoides tornieri* (lecithotrophic; Fig. 27). Unpigmented embryos also occur in the nidicolous *Altiphrynoides malcolmi* (Grandison 1978) and *Blythophryne beryet* (Chandramouli et al. 2016) and the direct developer *Oreophrynella nigra* (McDiarmid and Gorzula 1989), and these cases may represent three additional independent transformations. Regarding *Pedostibes tuberculosus*, Dinesh and Radhakrishnan (2013) reported the occurrence of a few, unpigmented embryos, but Chan et al. (2016) described and figured numerous pigmented eggs without further comments. Finally, numerous other species of bufonids have unpigmented eggs (e.g., *Ansonia* spp., Inger 1960a; *Frostius pernambucensis*, Cruz and Peixoto 1982; *Laurentophryne parkeri*, Tihen 1960; *R. acrolopha* group, Trueb 1971), and the evolution of lack of egg pigmentation in Bufonidae is expected to be complex.

Sizes of the embryos at the tail-bud stage are also highly variable (see Appendix). Embryos of *Atelopus* (about 2 mm long) and *Bufotes viridis* (mean = 2.2 mm) are small. Embryos of *Melanophryniscus alipioi* (3.5 mm long) have about four times the volume of those of *M. krauczuki* and *M. sanmartini* (mean = 1.94 and 1.76 mm long, respectively), and embryos of *M. rubriventris* are almost 1.5 times larger than those of *M. atroluteus* of the same species group (mean = 3.08 and 2.38 mm long, respectively). Embryos of *Rhinella* cf. *cerradensis* and *R. rumbolli* are the largest (mean = 4.05 and 4.2 mm long, respectively), and embryos of the *R. granulosa* group are the smallest of the genus (2.4–2.7 mm). The size of the eggs is a good predictor of the embryo size in *Rhinella*; *R.* cf. *cerradensis*, *R. ornata*, and *R. rumbolli* have the largest eggs, and species of the *R. granulosa* group have the smallest (Pereyra et al. 2015). Likewise, the small eggs of *Atelopus* range from 0.8 to 2.2 mm (Lescure 1981; Karraker et al. 2006; Gawor et al. 2012). Masses of eggs of *Melanophryniscus* in streams and ponds contain hundreds of small eggs (33–70 in *M. moreirae* to 294–401 in *M. krauczuki*; egg diameter about 2 mm; Bustos Singer and Gutiérrez 1997; Bornschein et al. 2015), but phytotelm-dwelling species of *Melanophryniscus* and *Dendrophryniscus* lay small groups of large eggs in successive ovipositions (1–9 eggs per oviposition; egg diameter about 3 mm; de Carvalho 1949; Langone et al. 2008; Bornschein et al. 2015).

In most cases, egg size is positively correlated with tadpole and adult sizes (Fig. 3). Small embryos of *Melanophryniscus atroluteus*, *M. krauczuki*, and *M. sanmartini* develop into small tadpoles and small adults, and larger embryos of *M. macrogranulosus* and *M. rubriventris* correspond to the largest adults within the genus. Conversely, the large embryos of *Dendrophryniscus* aff. *berthalutzae*, *M. alipioi*, and *M. milanoi* develop into the smallest adults of our sample. As embryos of the *Rhinella granulosa* and *R. marina* groups grow larger, the size of tadpoles and adults also increase significantly, and *R.* cf. *cerradensis* has the largest embryos, tadpoles, and adults. With embryos about

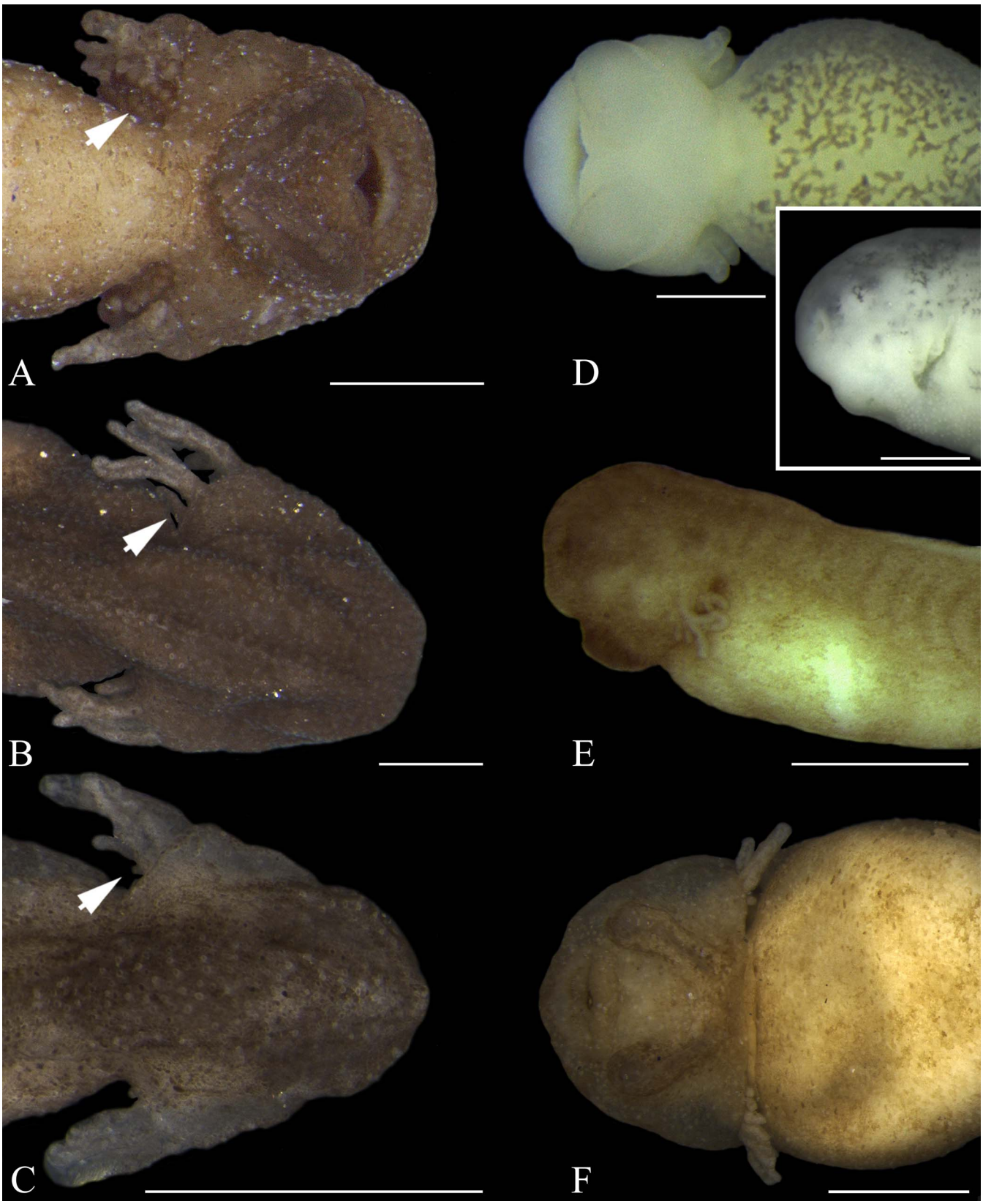


FIG. 4.—Structural differences of the external gills at maximum development in Bufonidae. Long third pair of gills: (A) *Bufotes viridis*; (B) *Rhinella arenarum*. Short third pair of gills: (C) *R. fernandezae*; (D) *Atelopus elegans* and detail of *A.* aff. *spumarius* at a comparable stage; (E) *Melanophryniscus krauczuki*; (F) *Dendrophryniscus* aff. *berthalutzae*. Scale lines = 1 mm. A color version of this figure is available on-line.

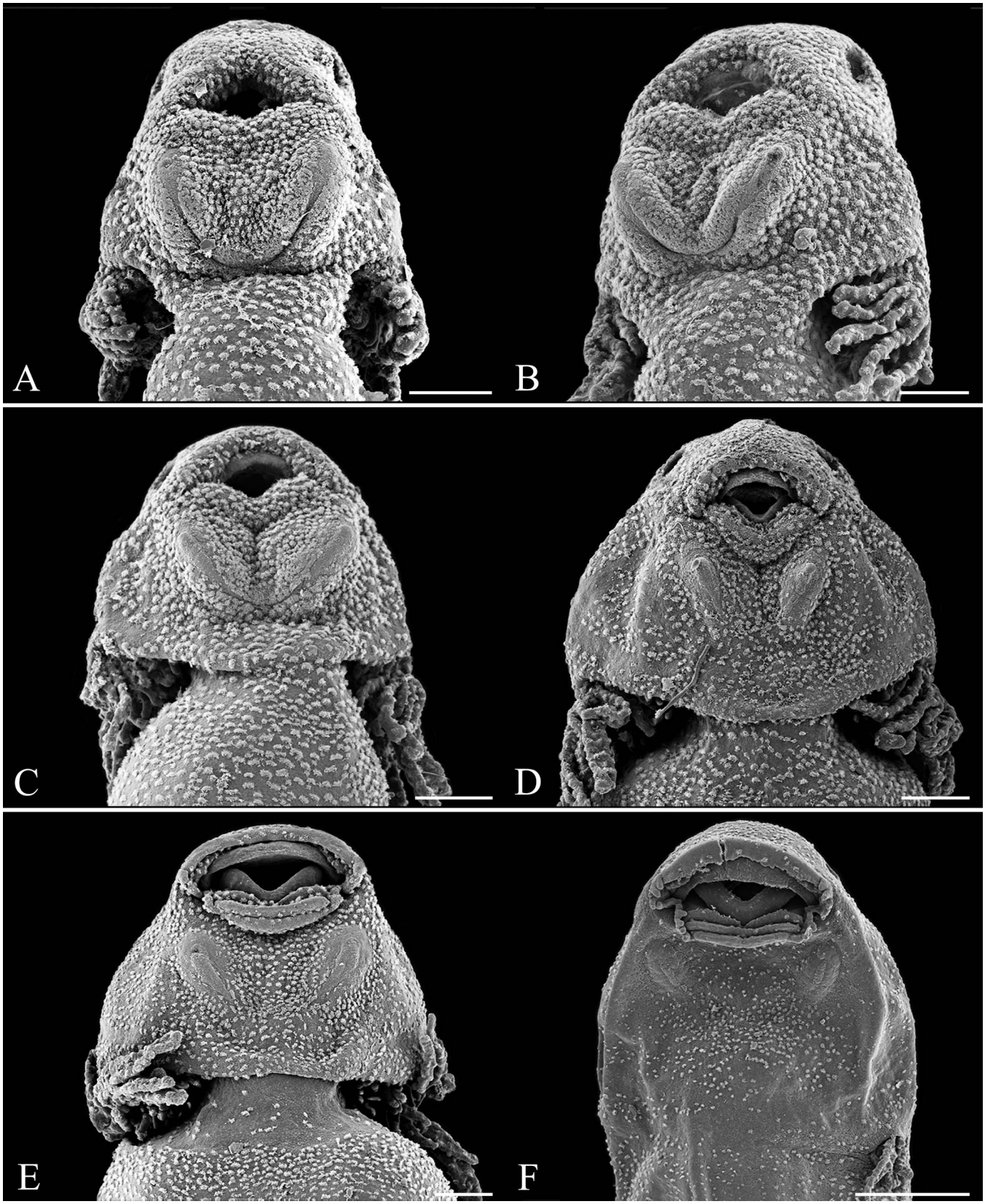


Fig. 5.—Ontogenetic changes in size and ciliation of the external gills and opercular development of *Rhinella ornata*. (A–C) Operculum at the gill base, (D,E) operculum medially fused, and (F) left gill regression. Scale lines = 200 μm.

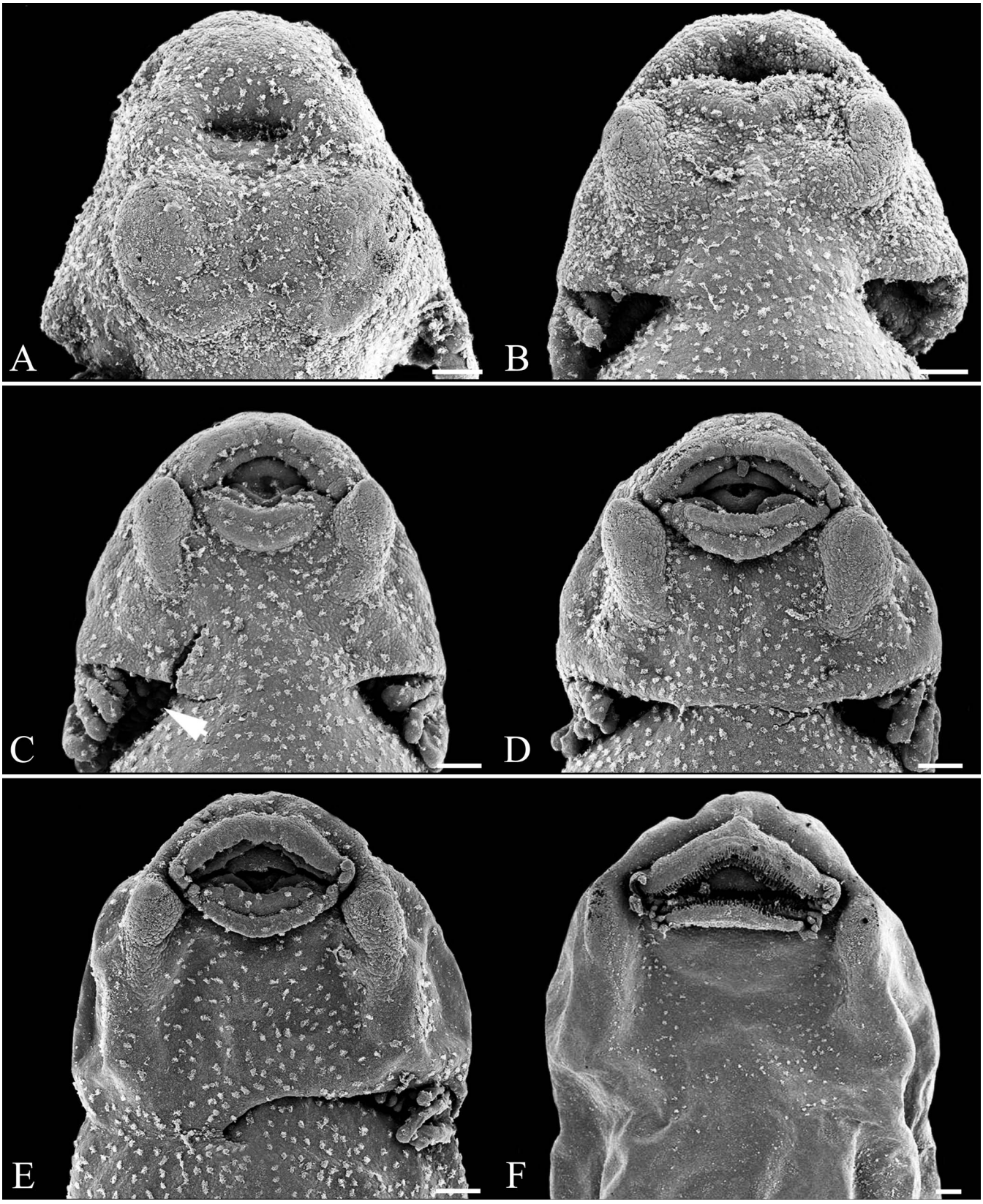


FIG. 6.—Ontogenetic changes in size and ciliation of the external gills and opercular development of *Melanophryniscus* aff. *devincenzii*. (A) First pair of gills branched, (B,C) operculum at the base of the gills, (D) operculum fused medially, (E) right gill concealed, and (F) spiracle complete. Note the small third gill pair visible ventrally (arrow). Scale lines = 100 μm.

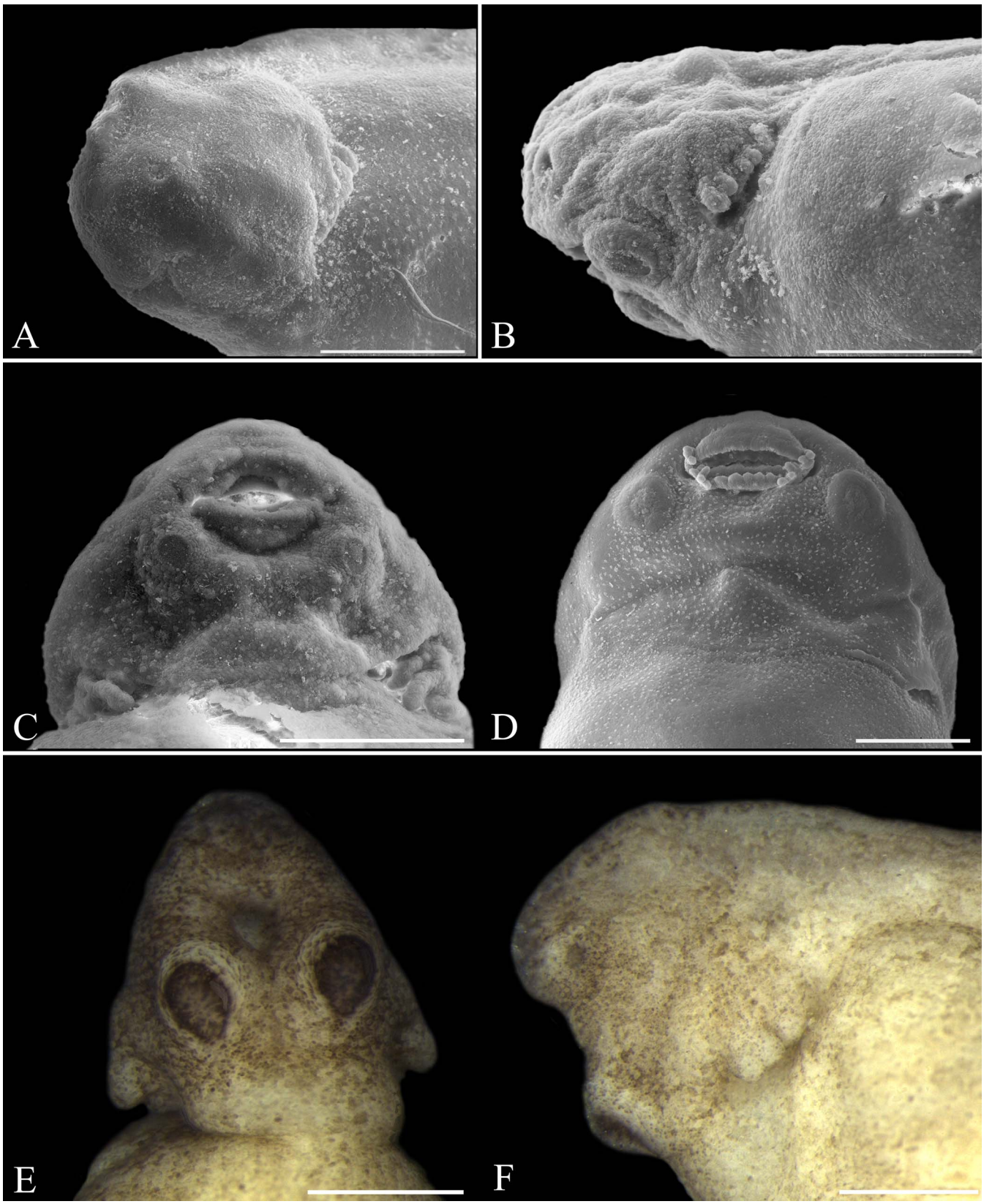


Fig. 7.—External gill development in species of the *Melanophryniscus moreirae* group. (A–D) *Melanophryniscus alipioi* embryos with two pairs of gills to spiracle complete, (E,F) *M. milanoi* at two pairs of gills and first pair branched stages. Note the two pairs scarcely developed in both species. Scale lines = 500 μm. A color version of this figure is available on line.

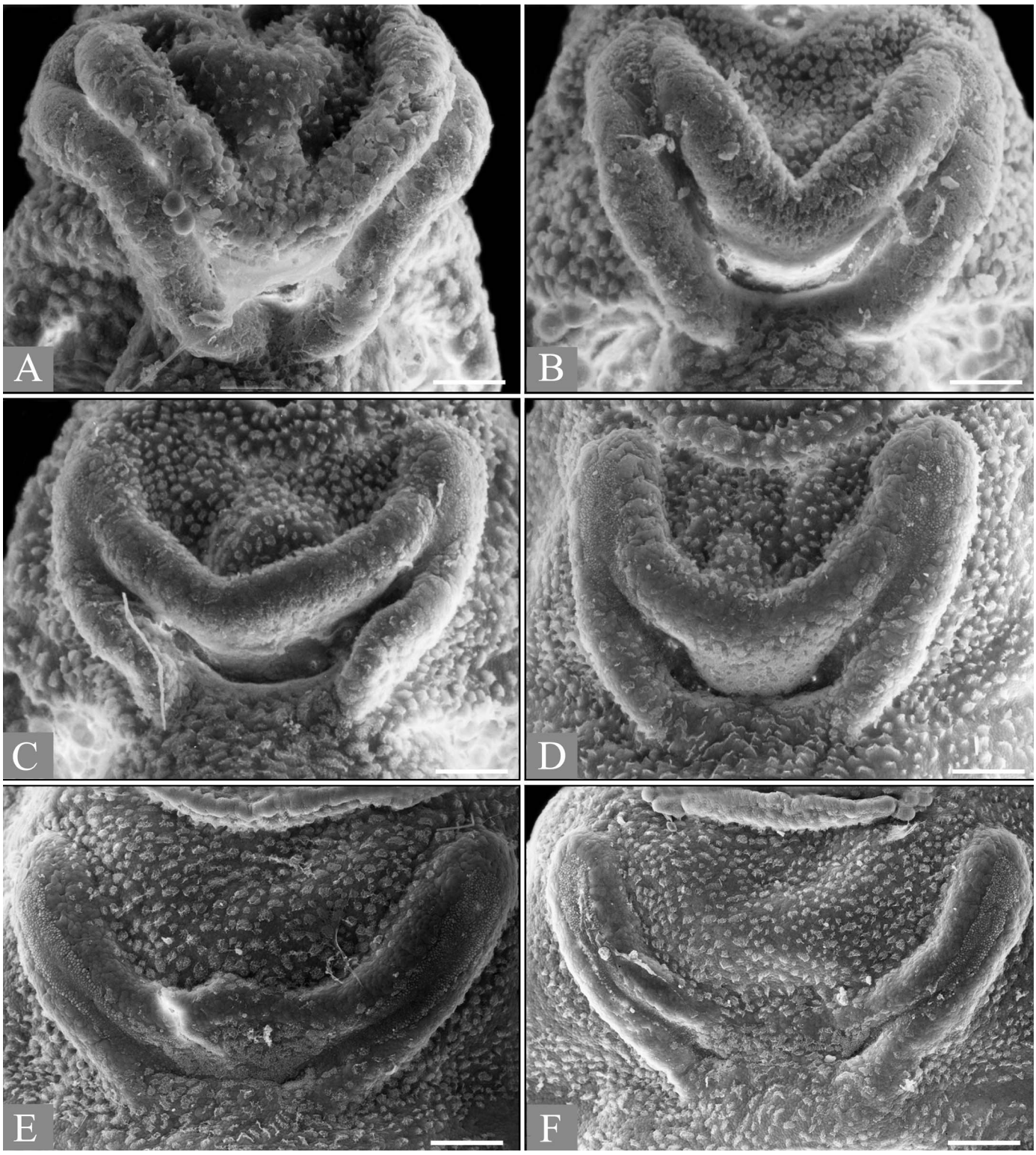


Fig. 8.—Development of adhesive glands of *Rhinella* cf. *cerradensis*. Glands in a morphogenetic pattern Type B begin with a deep V groove that persists long in ontogeny until the structure divides into two oblong halves. (A) Second pair of gills branched, (B,C) operculum at the base of the gills, (D) operculum medially fused, (E) right gill regressing, and (F) right gill concealed. Scale lines = 100 μm.

the size of those of *R.* cf. *cerradensis*, adults of *R. achalensis* and *R. rumbolli* are smaller than *R. arenarum* adults. *Atelopus* has values similar to those of the *R. granulosa* group species, whereas *Bufotes viridis* is similar to *R. arenarum*. In a study of the relationship between egg size, body (adult) size, and reproductive mode, Salthe and Duellman (1973) found that species with the same reproductive mode show a positive interspecific correlation between egg size and female body size, but if various reproductive modes are included, this correlation is inverted.

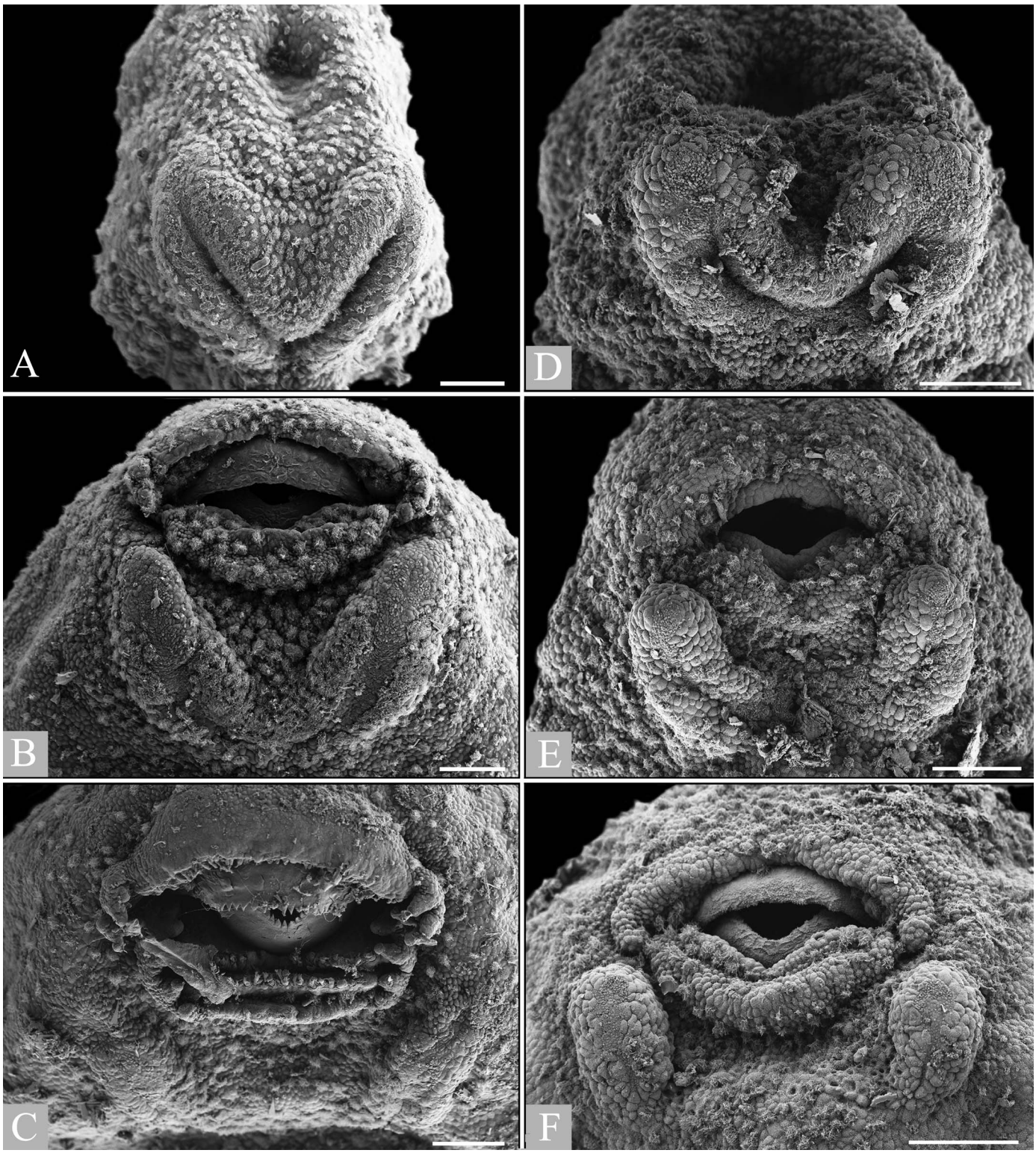


Fig. 9.—Structural variations in adhesive glands of Bufonidae. Morphogenetic Type B: *Bufotes viridis* embryos with (A) two pairs of gills, (B) right gill regressing, (C) left gill regressing. Morphogenetic Type A, where the initial groove has an M shape that soon splits into two ovoid structures: *Rhinella major* embryos with (D,E) operculum at the gill base and (F) operculum medially fused. Scale lines = 100 μm.

Likewise, in an analysis involving African bufonids, lecithotrophic viviparous forms show a positive correlation (Liedtke et al. 2014). Species with free-living larvae retain an inverse correlation of egg size with body size, but the authors attributed this to the heterogeneity of their data set, which includes explosive pond breeders and terrestrial nest breeders. In our case a proper analysis should discriminate between species that breed in ponds, streams, and phytotelmata in order to interpret different patterns of egg size–adult size relationships.

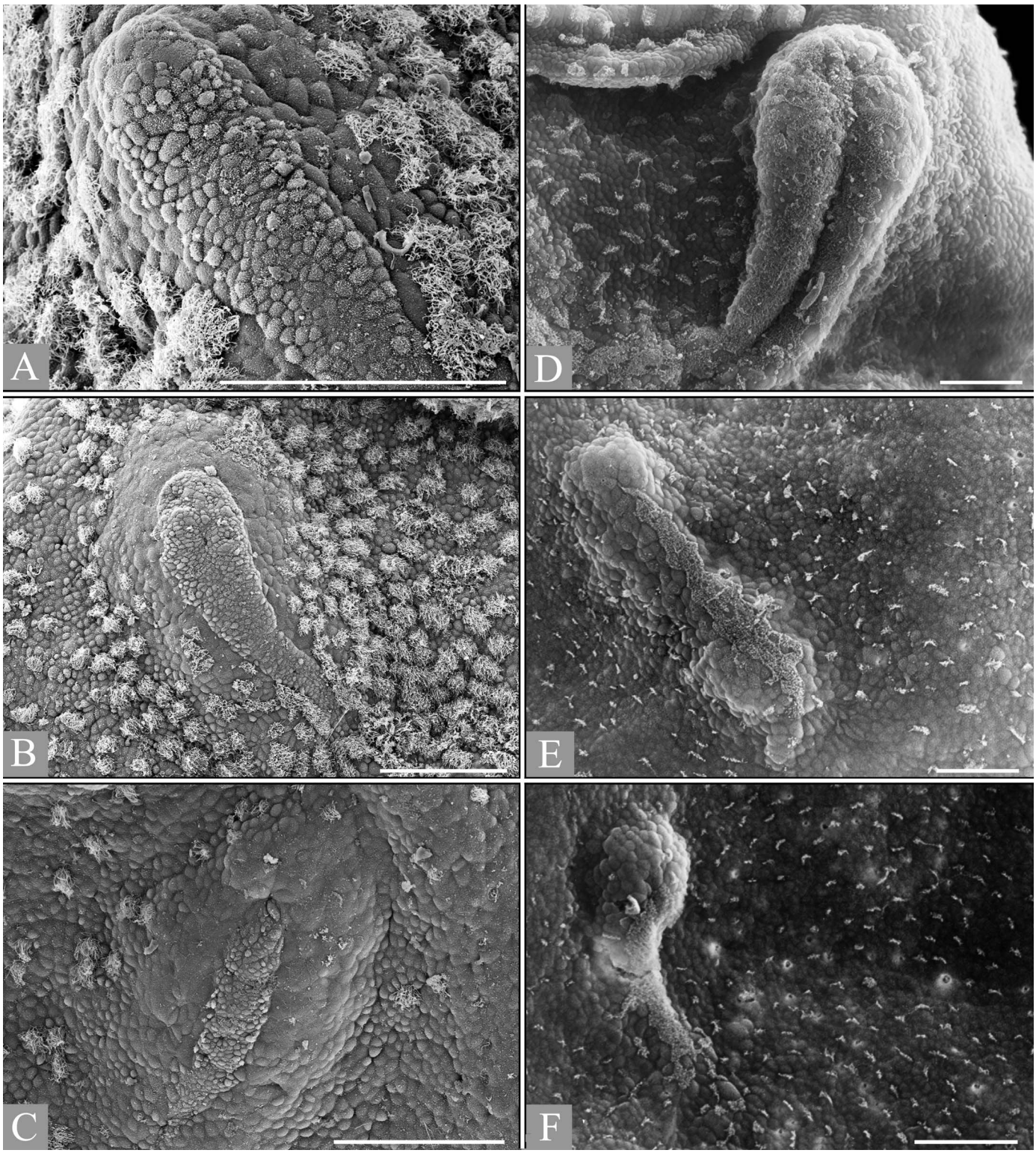


Fig. 10.—Details of shape and cell types of adhesive glands in Bufonidae. Morphogenetic Type B: *Rhinella ornata* embryos with (A) operculum at the gill base, (B) right gill regressing, and (C) left gill regressing. *Dendrophryniscus* aff. *berthalutzae* with (D) operculum medially fused, (E) spiracle complete, and (F) hind limbs twice as long as wide. Small secretory cells are clustered on the central region of the oblong glands. Scale lines = 100 μm.

The dorsal curvature of the body of embryos is not usually mentioned in studies of early development. In a comprehensive review, Richardson et al. (1997) illustrated a variety of amphibian tail-bud stages and commented on three states of dorsal curvature, i.e., kyphotic, lordotic, and straight, which were illustrated but not defined (and as we interpret, involuntarily mislabeled). Based on standard anatomical nomenclature, we understand kyphosis and lordosis as body curvatures over and away from the yolk mass, respectively. These curvatures may vary in their

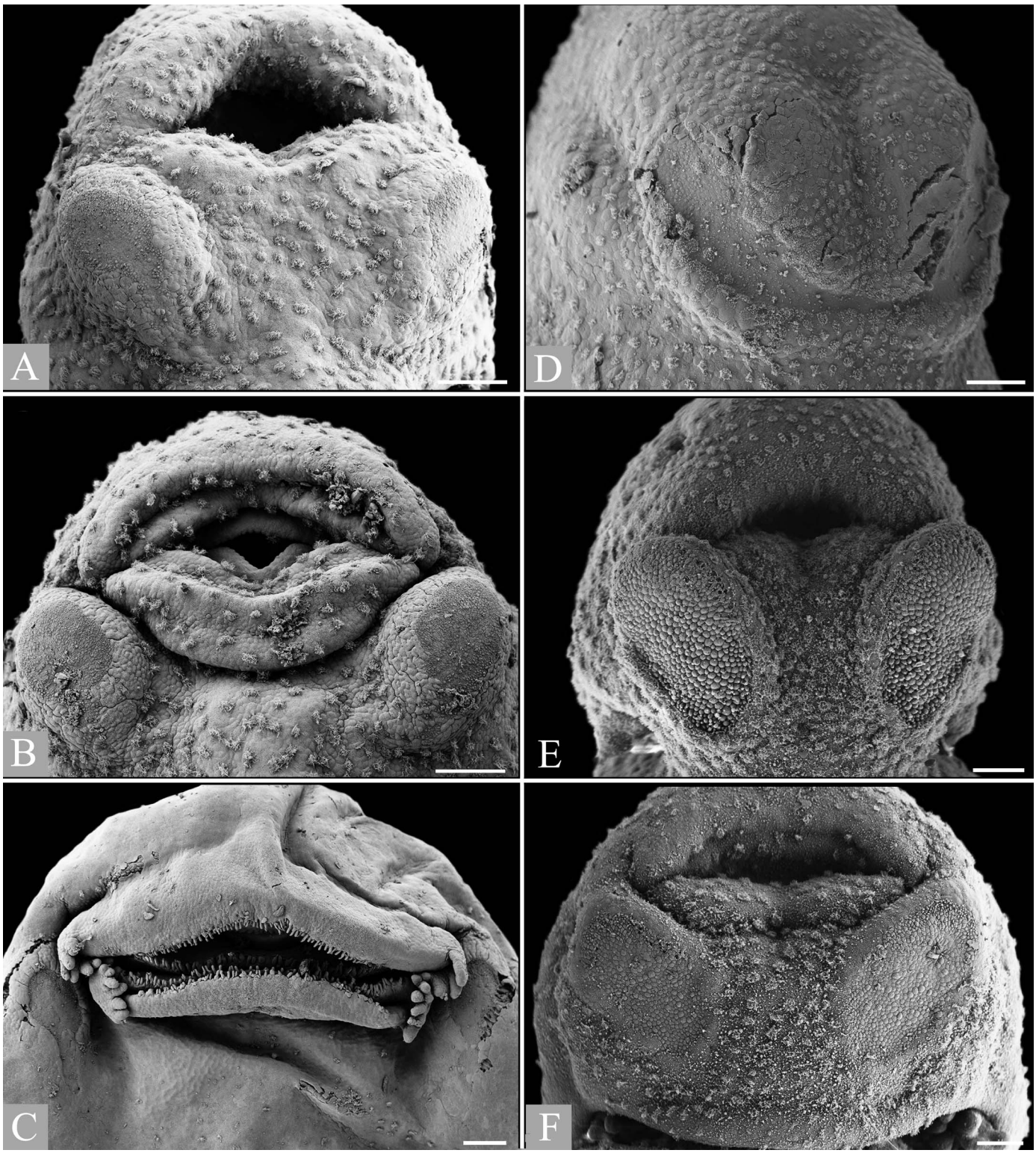


Fig. 11.—Structural variations in adhesive glands of Bufonidae. Morphogenetic Type Cs never form a V groove, but start as two large, rounded structures. *Melanophryniscus krauczuki* embryos with (A) operculum at the gill base, (B) operculum medially fused, and (C) spiracle complete. *Atelopus elegans* at (D) tail-bud stage, (E) first gill pair, and (F) operculum medially fused. Scale lines = 100 μm.

extent, including slightly kyphotic embryos that curve their heads or tail buds ventrally (e.g., *Pleurodeles*), embryos that curl completely over the yolk mass to produce an almost spherical aspect (e.g., *Siphonops*), slightly lordotic embryos with a subtle downward dorsal curvature (e.g., *Rana temporaria*), and strongly lordotic embryos where head and tail bud almost touch dorsally (e.g., *Pseudacris*). Straight embryos lack body curvature and show the typical lengthening as depicted for *Bufo bufo* and *Incilius valliceps*. In addition, the size and shape of the yolk mass contribute

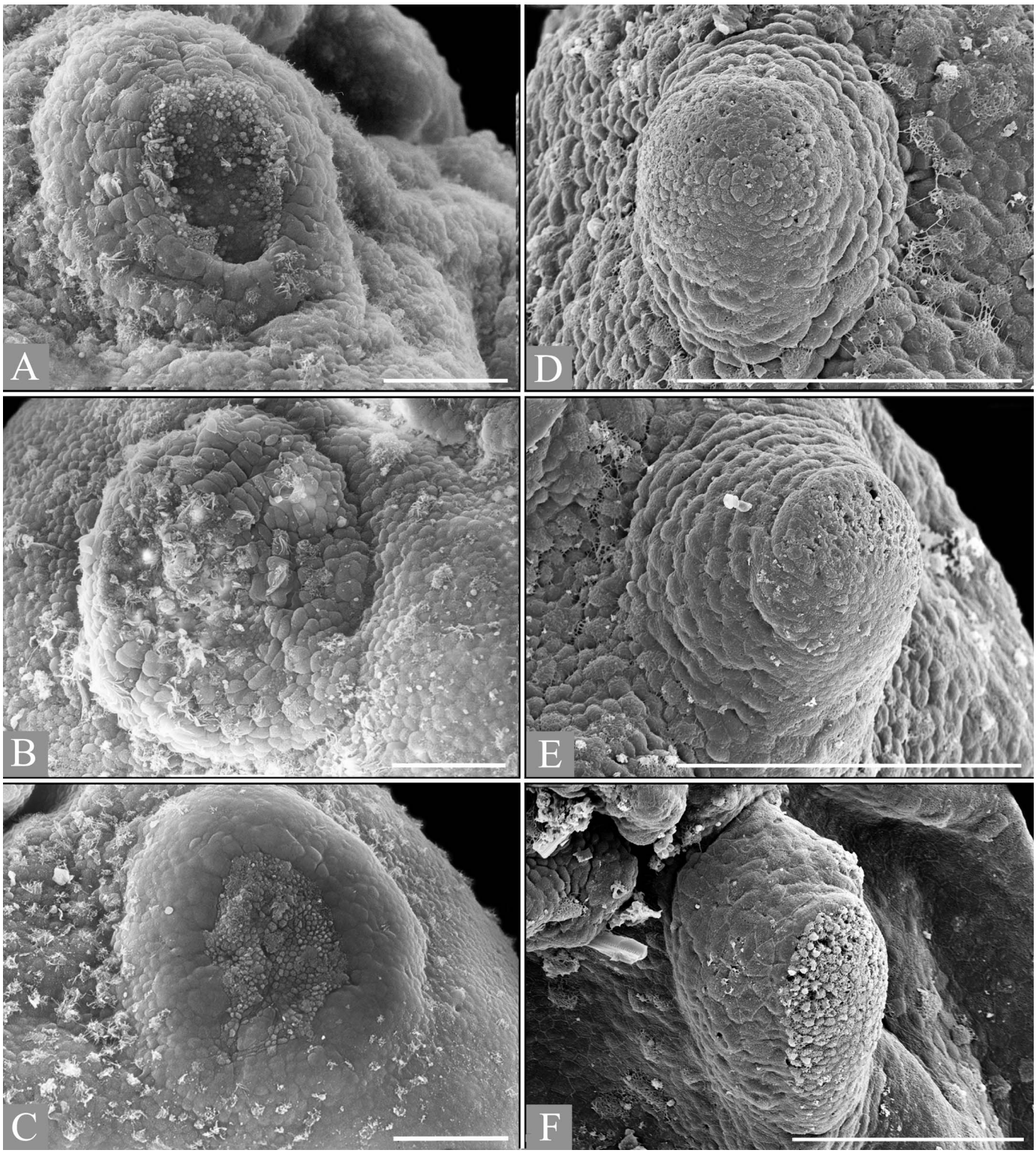


Fig. 12.—Details of shape and cell types of adhesive glands in Bufonidae. Morphogenetic Type C: *Melanophryniscus alipioi* embryos with (A) two pairs of gills branched, (B) left gill regressing, and (C) spiracle complete. *Melanophryniscus atroluteus* with (D,E) operculum at the gill base, and (F) right gill regressing. Small secretory cells are clustered on the central region of the rounded glands. Scale lines = 100 μm.

to the embryo shape. In most straight embryos the yolk is oblong and never projects beyond the head–tail ventral plane (e.g., *Incilius*). In other cases, a straight embryo develops on top of a large, spherical yolk mass (e.g., *Eleutherodactylus* spp. and *Epipedobates machalilla*; Townsend and Stewart 1985; del Pino et al. 2004) so that the overall configuration resembles a kyphotic embryo. In bufonids, these features differ among species (Fig. 2). In most forms, including *Bufo*, *Bufotes*, *Rhinella*, *Sclerophrys*, and several other derived genera (e.g., Sedra and Michael

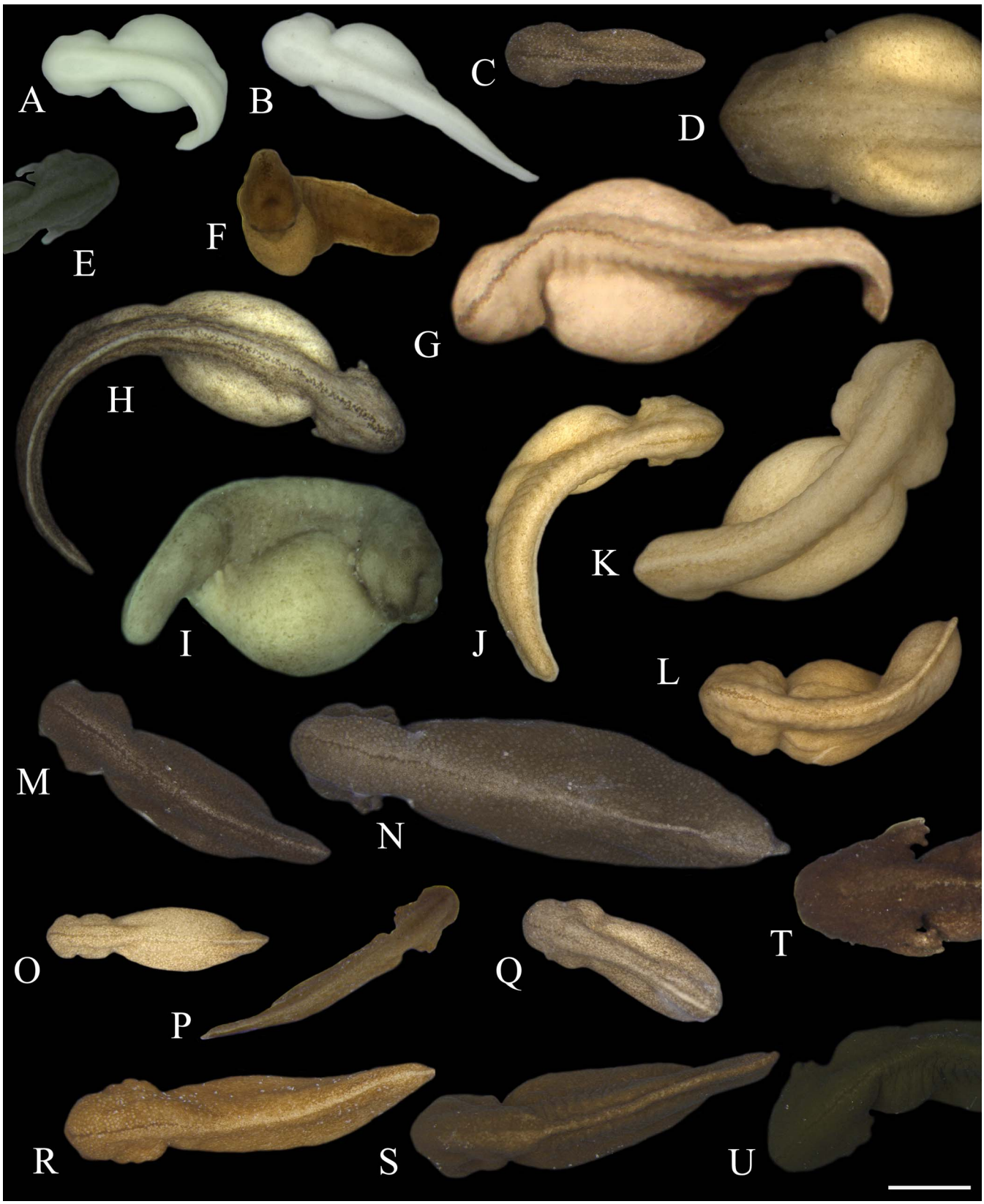


FIG. 13.—Hatching glands in Bufonidae. (A) *Atelopus elegans*, (B) *Atelopus*. sp., (C) *Bufotes viridis*, (D) *Dendrophryniscus* aff. *berthalutzae*, (E) *Melanophryniscus krauczuki*, (F) *M. sanmartini*, (G) *M. alipioi*, (H) *M. milanoi*, (I) *M. macrogranulosus*, (J) *M.* aff. *devincenzii*, (K) *M. atroluteus*, (L) *M. rubriventris*, (M) *Rhinella arenarum*, (N) *R.* cf. *cerradensis*, (O) *R. fernandezae*, (P) *R. major*, (Q) *R. azarai*, (R) *R. ornata*, (S) *R. rumbolli*, (T) *R. achalensis*, and (U) *R. spinulosa*. Note the long dorsal line in *M. alipioi* and *M. milanoi* embryos. Scale lines = 1 mm. A color version of this figure is available on line.

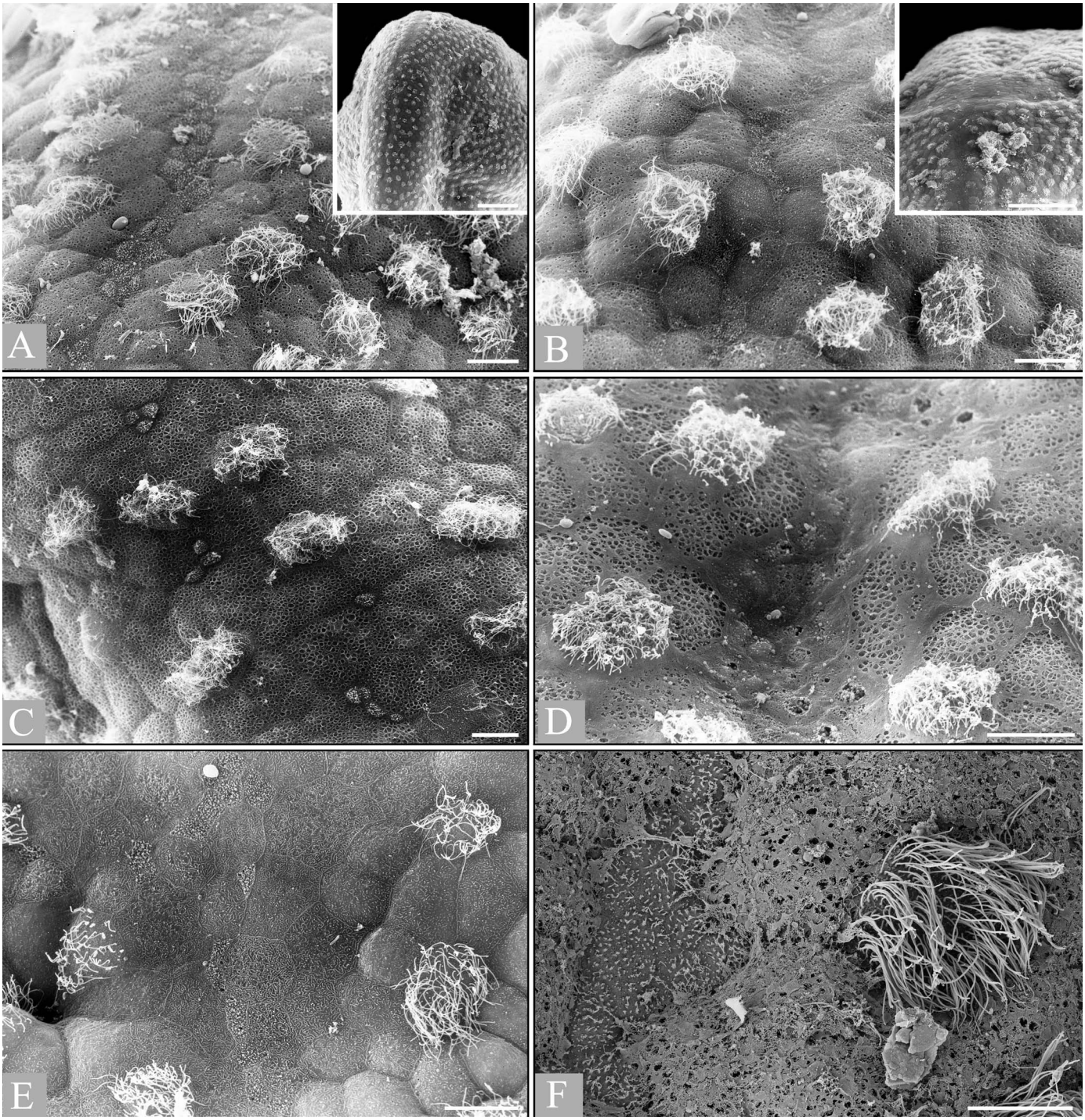


Fig. 14.—Variations in the arrangement of hatching gland cells in *Rhinella* embryos. *Rhinella arenarum* embryos with (A) branchial arches and (B) two pairs of gills. The insets depict a dorsal view of the cephalic region showing the general structure of the gland. (C) *Rhinella azarai* with branchial arches, (D) *R. ornata* with branchial arches, (E) *R. rumbolli* with branchial arches, and (F) *R. spinulosa* with the first pair of gills. Scale lines = 10 μm, insets = 100 μm.

1961; Richardson et al. 1997; Chandramouli et al. 2016; see Appendix), embryos are straight or slightly lordotic and strongly compressed, with scarce provision of the yolk never surpassing the ventral plane of the head–tail. Conversely, *Melanophryniscus* embryos have a kyphotic curvature, especially in *M. sanmartini* and *M. krauczuki* which curve tightly against the spherical yolk. *Atelopus* embryos are also kyphotic but not as markedly as those of *Melanophryniscus*. Our ancestral state reconstructions indicate the straight embryos and oblong yolk are derived conditions within Bufonidae (Fig. 27). Because of the lack of data in basal taxa, it is not possible to determine precisely the node where both transformations arose and whether their origins were independent. Although reversions to the plesiomorphic condition in the dorsal curvature do not occur in most derived clades within Bufonidae, two independent reversions to the spherical yolk occur in *B. aspinius* and *Pelophryne brevipes* and possibly in other

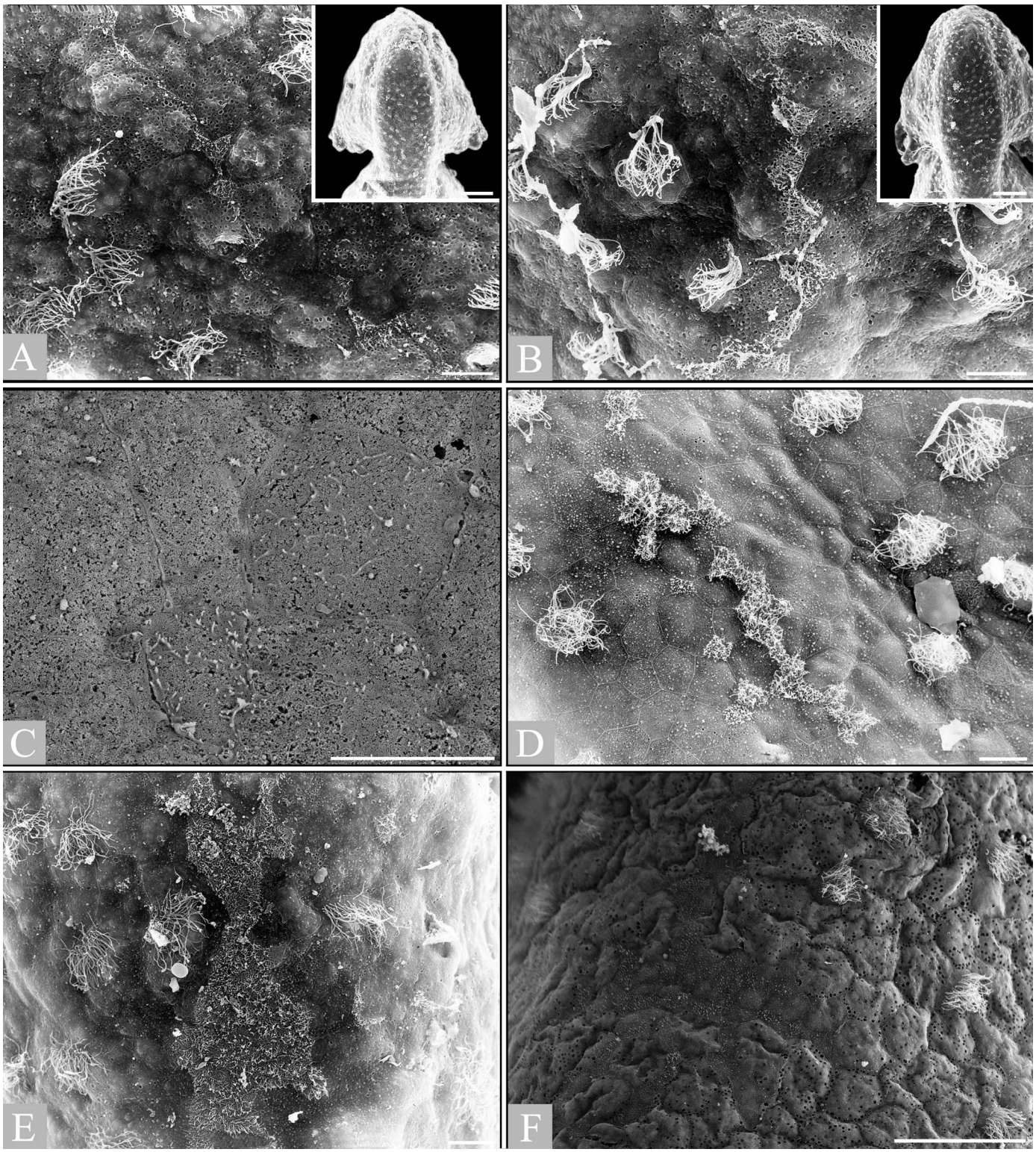


Fig. 15.—Variations in the arrangement of hatching gland cells in *Melanophryniscus* embryos. *Melanophryniscus atroluteus* embryos with (A) first gill pair branched and (B) operculum at the base of the gills. The insets depict a dorsal view of the cephalic region showing the general structure of the gland. (C) *Melanophryniscus rubriventris* with branchial arches, (D) *M.* aff. *devincenzii* with operculum at the base of the gills, (E) *M. krauczuki* with the first pair of gills, (F) *M. sanmartini* with branchial arches. Scale lines = 10 μm, insets = 100 μm.

endotrophic forms such as *Nectophrynoides* and *Nimbaphrynoides*.

Large, yolk-rich, kyphotic embryos are known in some anuran species in relation to oviposition in lotic environments (e.g., species of several genera of stream-breeding hylids and mantellids; Lang 1995; Faivovich et al. 2006; Scheld et al. 2013), oviposition outside of the water (e.g., some *Batrachyla*, *Hyalinobatrachium*, *Leptodactylus fuscus*

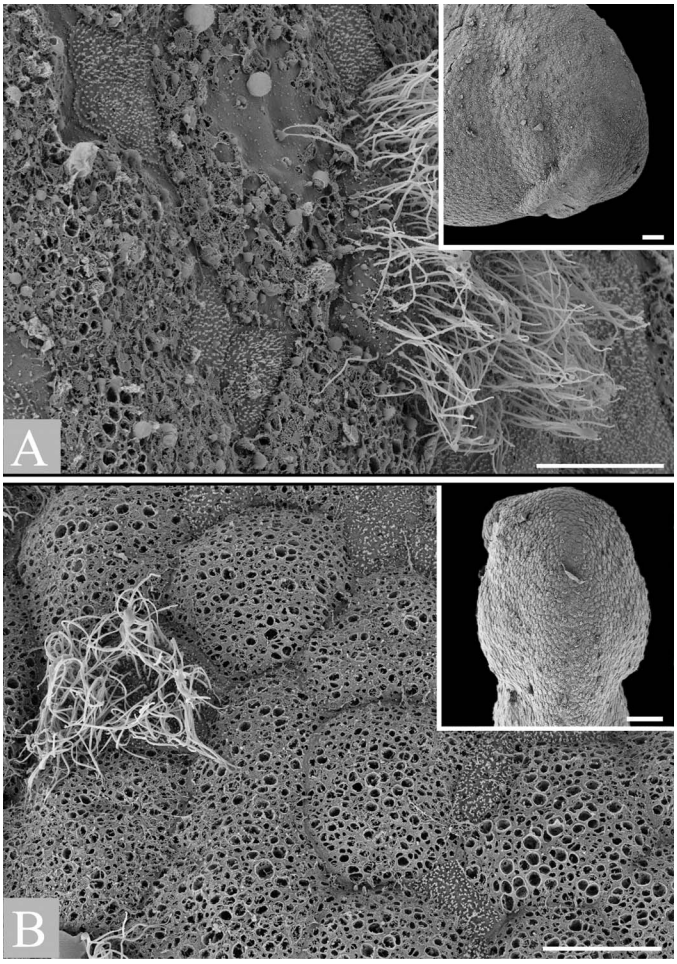


FIG. 16.—Hatching gland cells in other bufonid species. (A) *Atelopus* aff. *spumarius* at tail-bud stage and (B) *Bufotes viridis* with branchial arches. The insets depict lateral and dorsal views of the cephalic region to show the general structure of the glands. Scale lines = 10 μm, insets = 100 μm.

group, and *Phyllomedusa*; Salica et al. 2011; Salazar-Nicholls and del Pino 2015; Nokhbatolfoghahai et al. 2015; J. Grosso, personal observation), or those with endotrophic development (e.g., *Myobatrachus gouldii*, *Philoria sphagnicola*, *Platymantis vitianus*, and several Brachycephaloidea; Townsend and Stewart 1985; De Bavay 1993; Anstis et al. 2007; Nokhbatolfoghahai et al. 2010; Narayan et al. 2011; Goldberg and Vera Candioti 2015). In our study, a relationship with oviposition site is not always clear. *Melanophryniscus* embryos all show various extents of kyphotic curvature whether they develop in lotic (e.g., *M. krauczuki*) or lentic (e.g., *M. sanmartini*) environments. Likewise, all *Rhinella* embryos lack dorsal curvature independently of habitat type. Embryos of both *Atelopus* embryos are kyphotic and lotic.

Several kyphotic embryos, especially those with terrestrial and endotrophic development, hatch late so that the large yolk supply can guarantee a nutritional source during the long intracapsular period. Chipman et al. (1999) described developmental aspects of a small hyperoliid and discussed the correlation between kyphosis and egg size and noted that, unlike the small *Hyperolius puncticulatus* in their study (egg diameter = 1.5–1.8 mm), most known kyphotic embryos are large (e.g., *Ascaphus*, *Eleutherodactylus*, and *Gastrotheca*). Among the species we have examined, kyphotic embryos of *Melanophryniscus alipioi*, *M. macrogranulosus*, and *M. rubriventris* are larger than 3 mm (3.1–3.5 mm), but the remaining *Melanophryniscus* and *Atelopus* species (1.7–2.7 mm) range in sizes similar to those of *H. puncticulatus*. Kyphosis in this latter species is due to a pseudo-meroblastic cleavage that results in a large, spherical yolk mass and an embryo that curls across it (Chipman et al. 1999). Although we did not focus on a detailed study of gastrulation in *Melanophryniscus* and *Atelopus*, the configuration of the embryos is almost identical to that described in *Hyperolius*.

External Gills
(Figs. 4–7 and 26)

All the bufonid embryos that we studied develop three pairs of gills (except *Dendrophryniscus* aff. *berthalutzae*, *Melanophryniscus macrogranulosus*, and *M. sanmartini*, where we could not determine it). Differences pertain to the extent of development of main branches, ciliation, and developmental timing. In *Bufotes viridis* and most species of *Rhinella* (Figs. 4A,B and 5), three pairs of gills are clearly visible, and the first and second ones are comparatively longer and more branched. Fully developed gills range from 5.4 to 8.8% of the body length with the largest gills occurring in the embryos of *R. ornata*. The third pair appears as a thin, scarcely branched bar that at full development reaches half the length of the other pairs. In species of the *R. granulosa* group, the gills are large (6.2–7.8% of the body length), but the third pair is very short and never extends beyond the opercular margin (Fig. 4C). In *Atelopus* and *Melanophryniscus*, two pairs of gills are visible externally, and the third one is very small and late to develop so that it is always concealed by the operculum. In *Atelopus* (Fig. 4D), the gills are only 3.8% and 1.4% of the body length in *A. elegans* and *A.* aff. *spumarius*, respectively. In *Melanophryniscus* (Figs. 4E, 6, and 7) the gills are generally larger than in *Atelopus*. The two first gills range from 1.8–7% of the body length at maximum development and are comparatively longer in *M. atroluteus* and *M. rubriventris* (6.8–7%). Gills of *M. alipioi* and *M. milanoi* are particularly small and transient (about 1.8%; Fig. 7). Two pairs of gills were described for *Bufo bufo*, *R. humboldti*, and *R. marina* (Nokhbatolfoghahai and Downie 2008), and three pairs were reported in *Bufotes oblongus* (Dujsebayeva et al. 2004) and *Barbarophryne brongesrmai* (Grillitsch et al. 1972). Four pairs of gills were described in *B. viridis* (Nokhbatolfoghahai and Downie 2008) and *Duttaphrynus melanostictus* (Khan 1965). In our ancestral state reconstruction, the wide distribution of a short or absent third gill pair may be misleading because of the lack of specific mentions or detailed observations in some literature sources. The occurrence of a third pair of gills extending beyond the opercular margin optimizes as a synapomorphy of *Rhinella* within Bufonidae with three instances of homoplasy in *B. brongersmai*, *Bufotes viridis*, and *D. melanostictus* (Fig. 27). Likewise, a very short third pair of gills is a synapomorphy of the *R. granulosa* group with an instance of homoplasy in *R. marina*.

Gill ciliation also varies; *Rhinella ornata* (Fig. 5), *Bufotes viridis*, *R.* cf. *cerradensis*, and *R. rumbolli* embryos have the densest ciliation, and gills of *Atelopus* almost lack ciliated cells. Gills of *Melanophryniscus* generally have intermediate ciliation. Likewise, bufonid embryos studied by Nokhbatol-

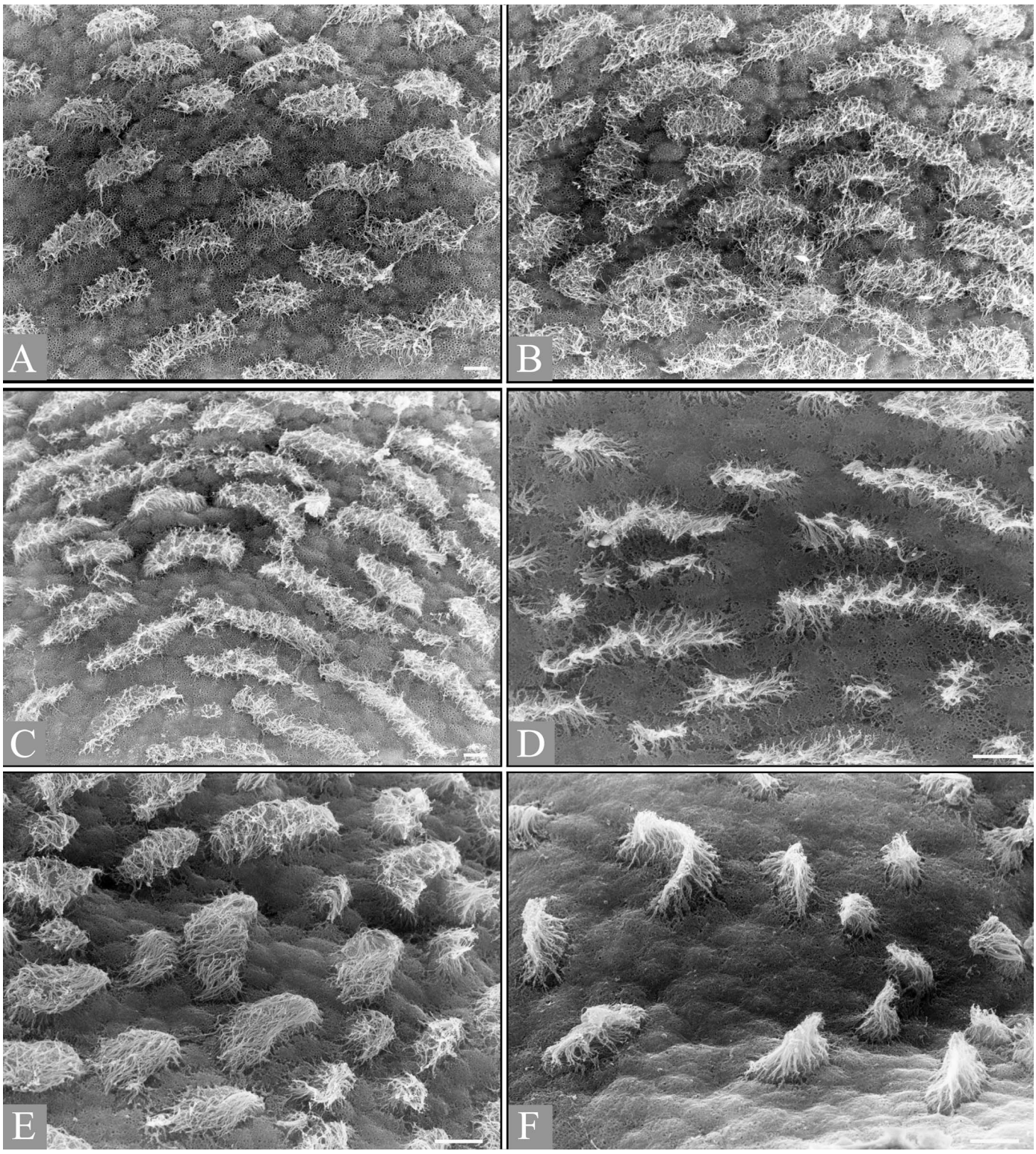


Fig. 17.—Development of body ciliation in the abdominal region of *Rhinella* cf. *cerradensis* embryos with (A) second pair of gills branched, (B) operculum at the base of the gills, (C) gills at complete development, (D) operculum medially fused, (E) right gill regressing, and (F) spiracle complete. Scale lines = 10 μm.

foghahai and Downie (2008) show intermediate to dense ciliation of the external gills.

Besides structural variation, some heterochronic changes are evident during gill development in bufonids (Table 1; Fig. 26). The most diverging species are those of *Atelopus*, where gill buds appear and branch later than in the remaining taxa after the operculum has differentiated. Gill development is delayed in most *Melanophryniscus* relative to *Bufotes* and *Rhinella*. The two latter genera share an initial developmental sequence similar to the reference

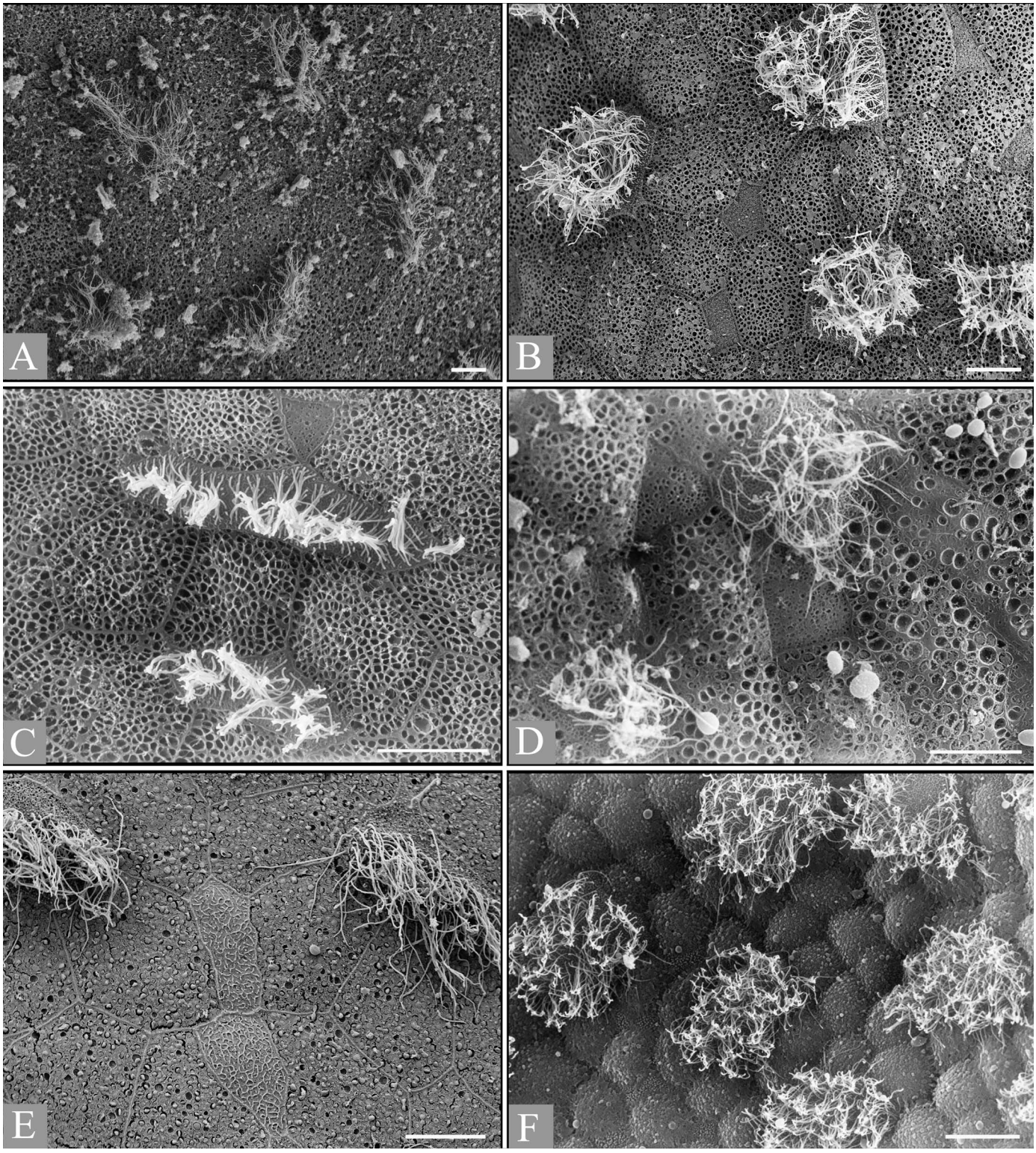


FIG. 18.—Variation in ciliated cell shapes in Bufonidae. (A) *Atelopus elegans* embryos with the operculum medially fused, (B) *Bufotes viridis* with branchial arches, (C) *Dendrophryniscus* aff. *berthalutzae* with the spiracle formed, (D) *Melanophryniscus alipioi* with the second pair of gills branched, (E) *M. krauczuki* with the operculum at the base of the gills, and (F) *Rhinella rumbolli* with the operculum medially fused. Scale lines = 10 μm.

trajectory and diverge on the late differentiation of the third pair of gills. Complete gill development is reached before the operculum fuses medially, except in *R. major*, *R. ornata*, *R. rumbolli*, and *R. spinulosa*, in which the events are inverted.

Adhesive Glands
(Figs. 8–12 and 26)

Adhesive glands are transient structures that produce mucus that allows embryos to adhere to surfaces inside and outside the egg. They form very early after neurulation as a

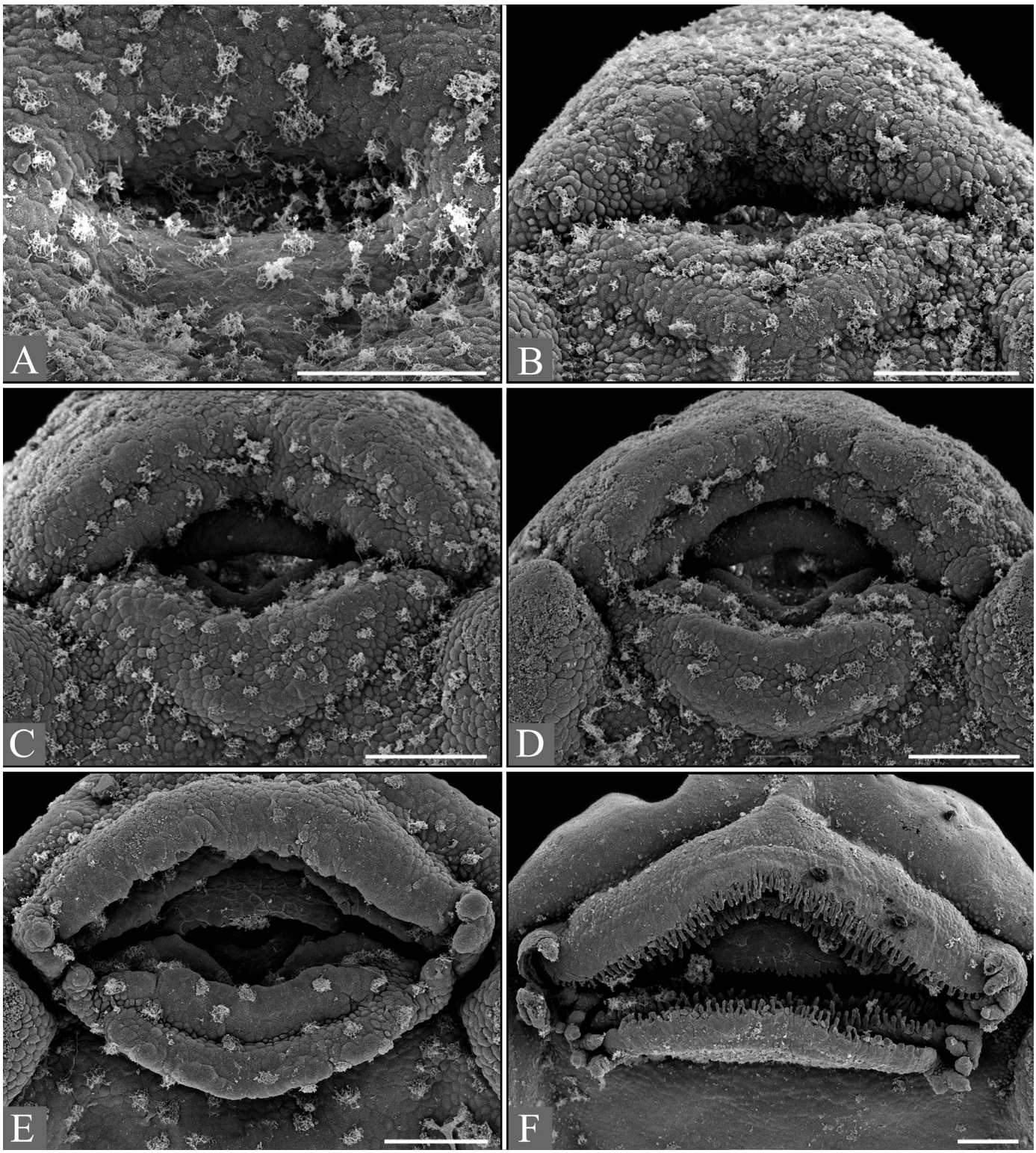


Fig. 19.—Development of the oral disc in *Melanophryniscus* aff. *devincenzii*. Embryos with (A) second pair of gills present, (B) second pair of gills branched, (C,D) operculum at the base of the gills, (E) right gill concealed, and (F) spiracle formed. Scale lines = 100 μm.

transverse field of cells just posterior to the mouth. Nokhbatolfoghahai and Downie (2005) described the adhesive glands of a heterogeneous group of species and recognized five morphogenetic patterns that relate to taxonomy and ecomorphology. Three bufonids examined in that paper, *Bufo bufo*, *Bufotes viridis*, and *Rhinella marina*, share a morphological Type B gland that is characterized by a primordium that invaginates as a deep V-shaped groove and persists until later stages before it divides into two long branches. Likewise, *B. viridis*,

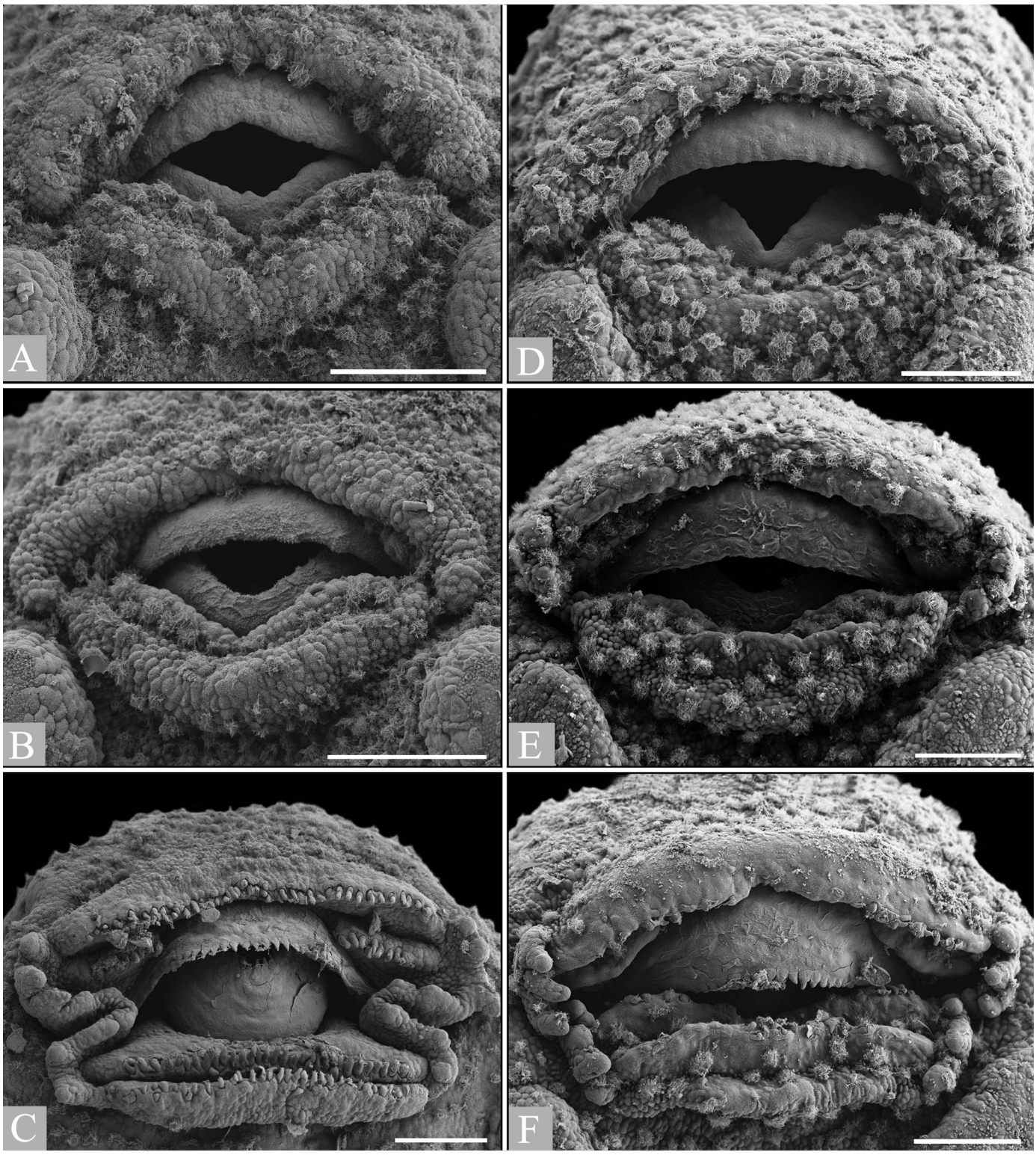


Fig. 20.—Variations in oral disc development in Bufonidae. *Rhinella major* embryos with (A,B) operculum medially fused and (B) spiracle complete. *Bufotes viridis* with (D) operculum at the gill base, (E) right gill regressing, and (F) left gill regressing. Scale lines = 100 μm.

Dendrophryniscus aff. *berthalutzae*, and most *Rhinella* (from the *R. crucifer*, *R. marina*, *R. spinulosa*, and *R. veraguensis* groups) in our study have Type B glands (Figs. 8 and 9A) with supporting cells that have microridges or cilia and secretory cells with microvilli dispersed along the central groove (Fig. 10). At full development the gland almost reaches the edge of the operculum (average about 15% of the body length). The gland in *R. rumbolli* differs from the others by having a wide U shape at first, and when the branches separate they remain more lateral and distant

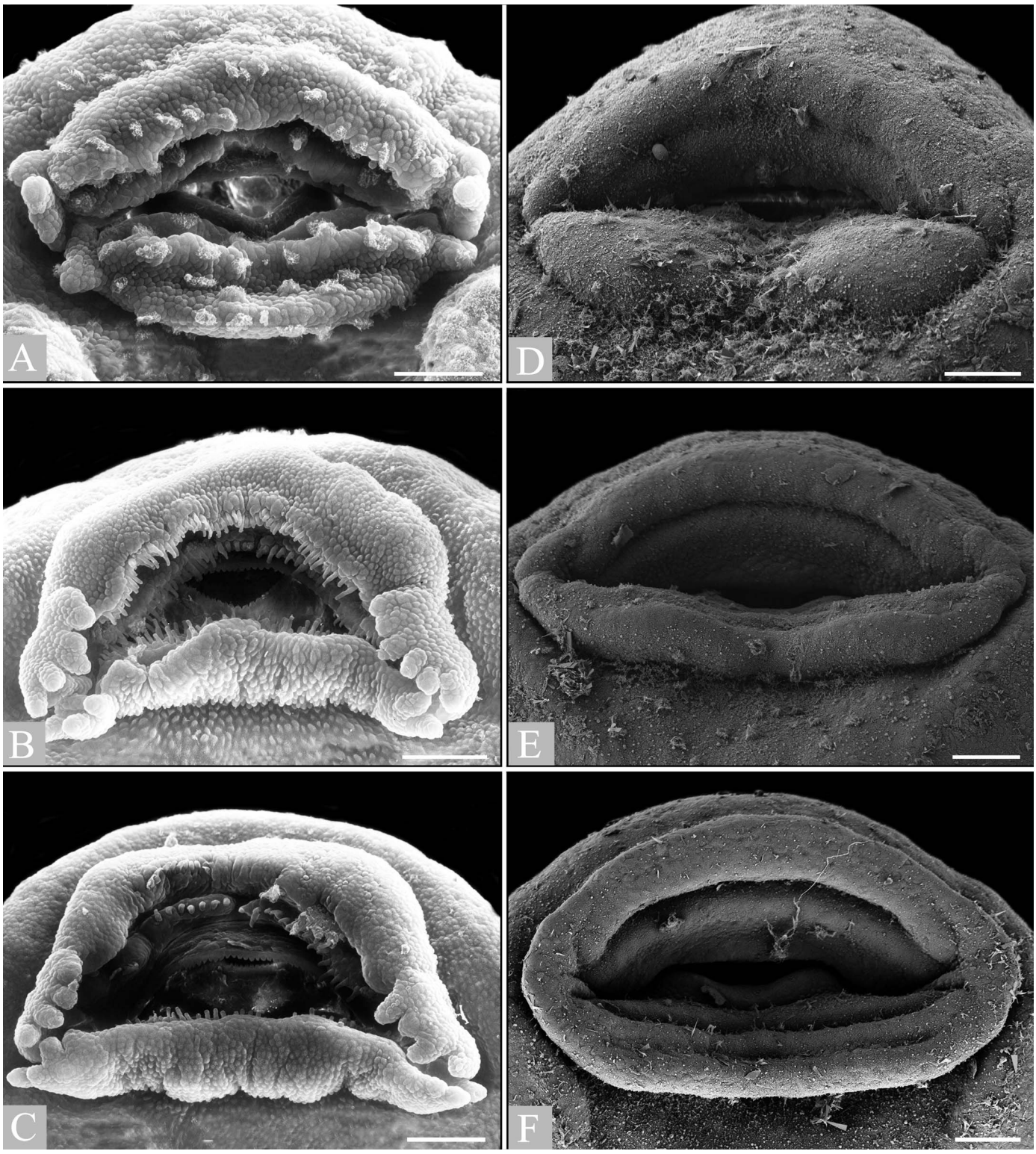


Fig. 21.—Variations in oral disc development in Bufonidae. *Dendrophryniscus* aff. *berthalutzae* embryos with (A) operculum medially fused, (B) spiracle complete, and (C) hind limb twice longer than wide stages. *Atelopus* aff. *spumarius* with (D) right gill concealed and (E,F) left gill concealed. Scale lines = 100 μm.

from each other (Fig. 25). Species from other derived genera in Bufonidae, such as *Anaxyrus*, *Bufo*, *Duttaphrynus*, *Epidalea*, *Incilius*, *Phrynoidis*, and *Sclerophrys*, also have Type B glands (Boulenger 1897; Lieberkind 1937; Gosner and Black 1954; Limbaugh and Volpe 1957; Khan 1965; Iwasawa and Saito 1989; Rao and Yang 1994; Ba-Omar et al. 2004; Gomez-Mestre et al. 2006).

The fourth bufonid species included in Nokhbatolfoghahai and Downie (2005) was *Rhinella humboldti* (as *Bufo beebei*, from the *R. granulosa* group). In this case Type A

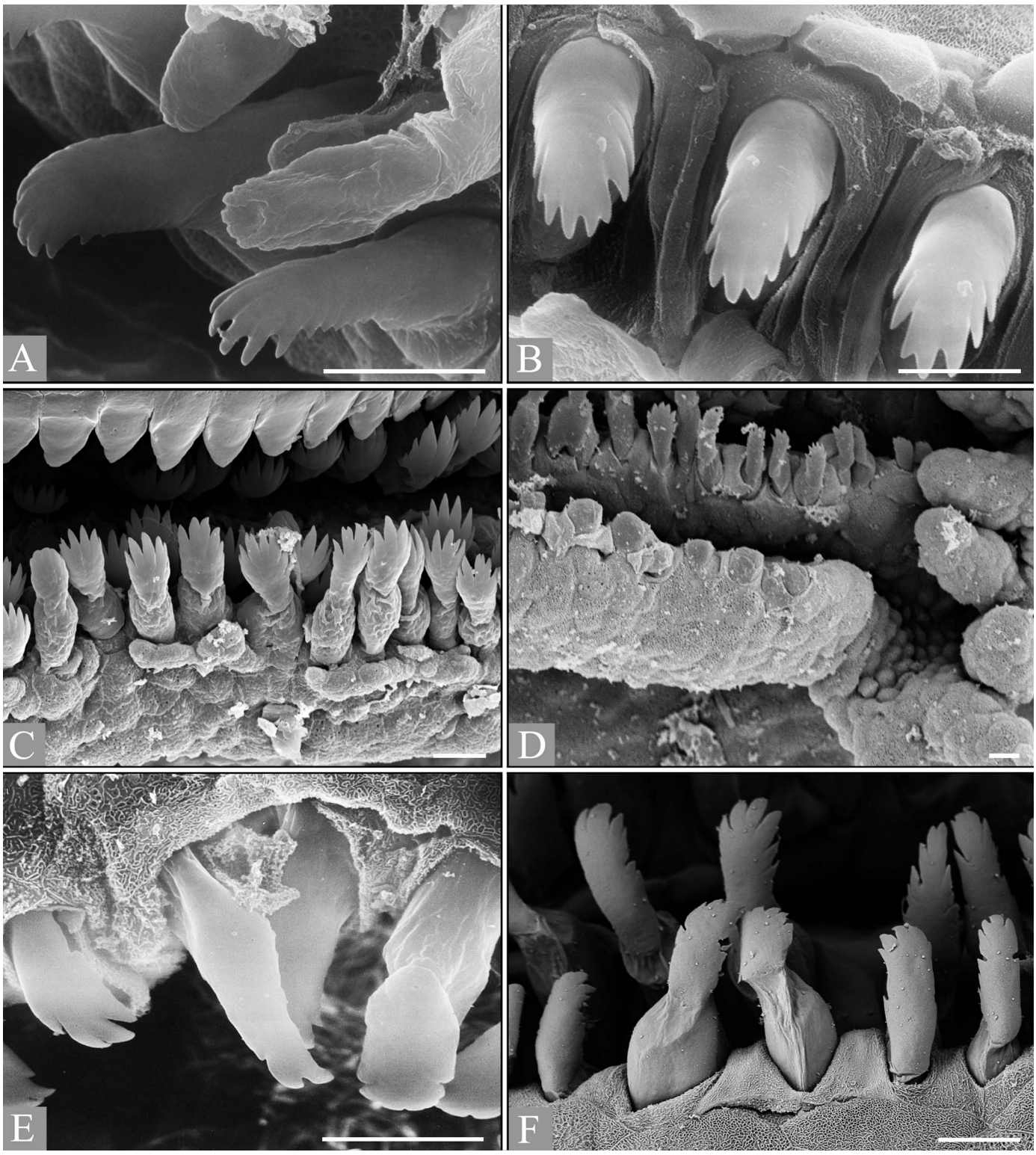


Fig. 22.—Comparative morphology of labial teeth in Bufonidae. (A) *Dendrophryniscus* aff. *berthalutzae* embryo with hind limb twice longer than wide, Row A1; (B) *Melanophryniscus alipioi* at Stage 31, Row A2; (C) *M. atroluteus* with spiracle complete, Rows P1–3; (D) *M.* aff. *devincenzii* with spiracle complete, Rows P2–3; (E) *M. macrogranulosus* with spiracle complete, Row P3; (F) *M. krauczuki* with spiracle complete, Rows P2–3. Scale lines = 10 μm.

glands are defined and characterized by an M-shape that divides into two oval structures with a central anterior–posterior groove that later disappears. The three species of the *R. granulosa* group we studied have this configuration (Fig. 9D–F). At its greatest development extent, the gland does not reach the edge of the operculum (about 12% of the body length). The main difference relative to Type B glands is that glands in the *R. granulosa* group subdivide earlier and become progressively smaller so that the last structures before they disappear are small, ovoid, and posterolateral to

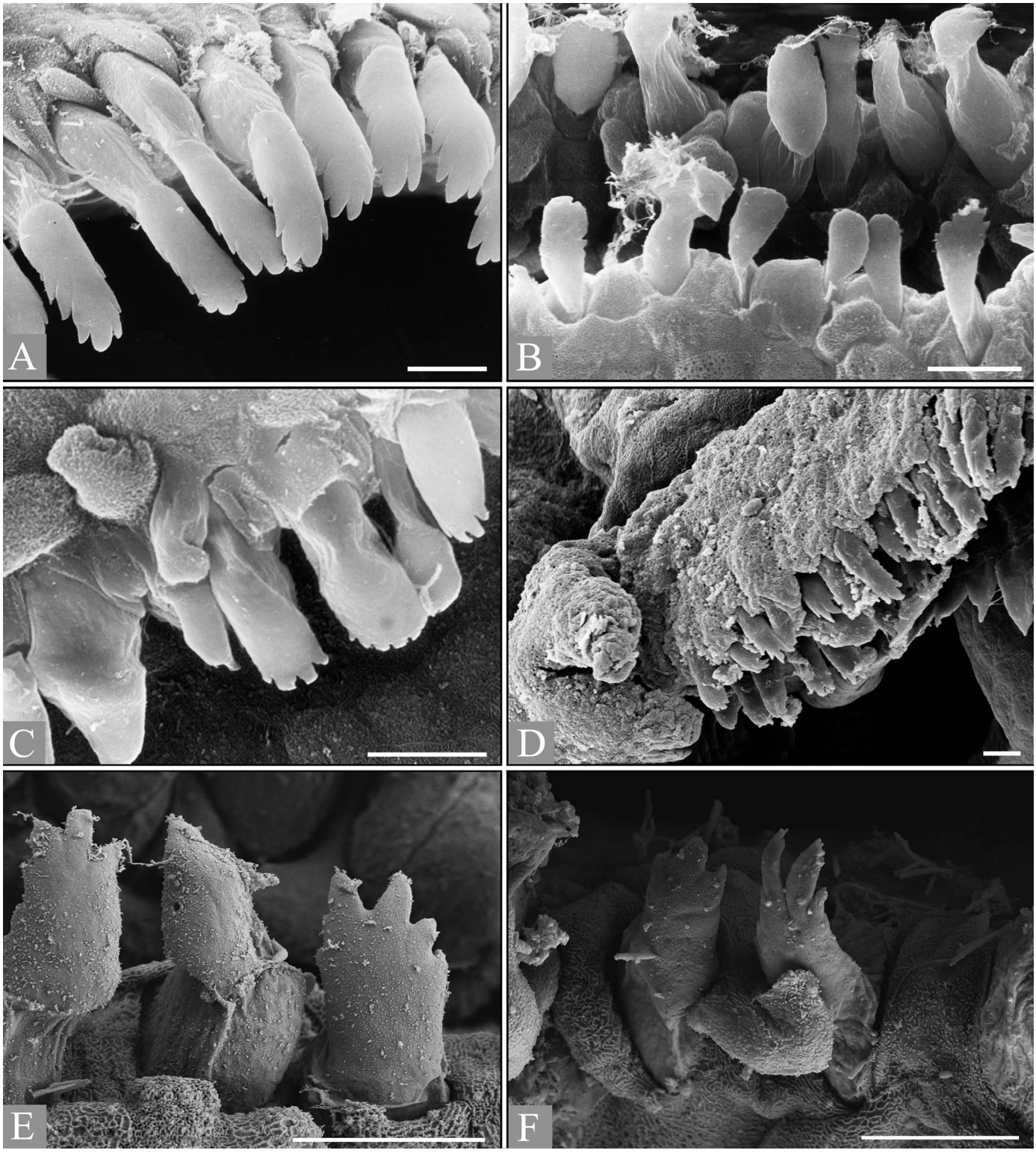


Fig. 23.—Comparative morphology of labial teeth in Bufonidae. (A) *Rhinella achalensis* embryo with spiracle complete, Rows A1; (B) *R.* cf. *cerradensis* with spiracle complete, Rows P2–3; (C) *R. rumbolli* with spiracle complete, Row A1; (D) *R. azarai* with spiracle complete, Rows A1–2; (E) *R. major* with hind limb as long as wide, Row P2; (F) *Bufotes viridis* with spiracle complete, Row P2. Scale lines = 10 μm.

the mouth. In the former group, a large V-shaped scar is still evident at older stages as the gland regresses without a significant size reduction.

Type C glands are present in *Atelopus* and *Melanophryniscus*. These glands lack a deep V-shaped groove, and the transverse field divides very early into two rounded structures that remain separated until regression. Interspecific variations mainly relate to size. The glands of *M. krauczuki* and the two species of the *M. tumifrons* group (Fig. 11A–C) are large and elongate at full development

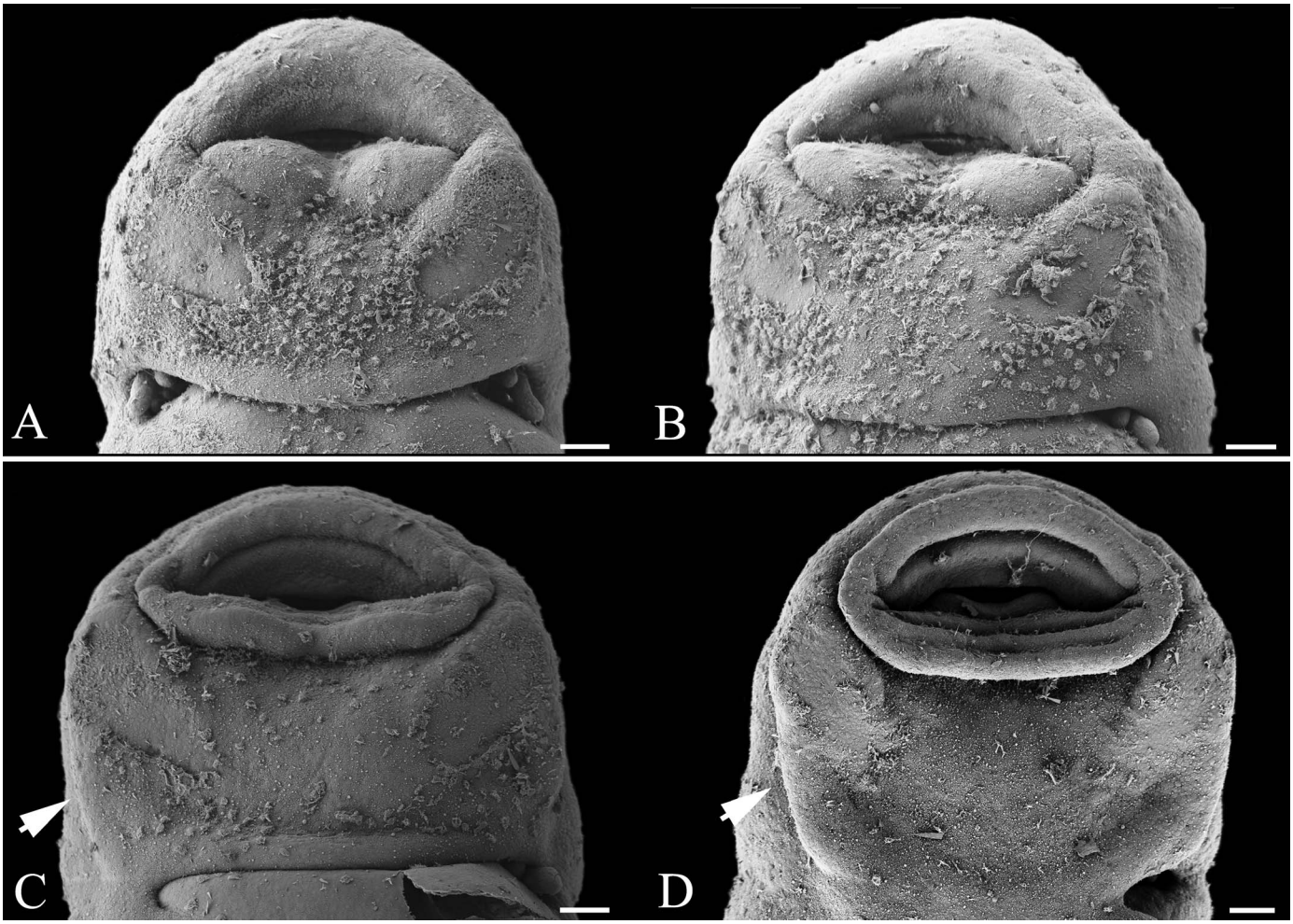


Fig. 24.—Initial development of the abdominal sucker of *Atelopus* aff. *spumarius*. (A) operculum fused medially, (B) right gill concealed stage, (C) right gill concealed stage, (D) left gill concealed stage. Note the longitudinal ridges delimiting the future lateral regions of the bulbous rim. Scale lines = 100 μm.

(about 15% of the body length). Conversely, glands in *M. sanmartini* and in the species of the *M. moreirae* and *M. stelzneri* groups are smaller and remain conical until they disappear (about 8% of the body length; Fig. 12). Glands in *Atelopus* (Fig. 11D–F) are enormous (13–19% of the body length); they are very close to each other at the beginning and separate as the oral disc widens.

Data on the distribution of types of gland in other Leptodacyliformes are scarce. Whereas Type C glands appear in leiuperines (Leptodactylidae; Nokhbatolfoghahai and Downie 2005), the glands in the bufonid sister group Dendrobatoidea are lacking in late-hatching embryos of *Dendrobates*, *Epipedobates*, and *Hyloxalus* (del Pino et al. 2004; Hervas et al. 2015). Our ancestral state reconstruction implies that the Type C glands are a synapomorphy of Bufonidae, with Type A and B adhesive glands being derived conditions within the family (see Fig. 27). Because there are no data on adhesive glands in *Osornophryne*, we cannot discern whether Type B glands are a synapomorphy of its sister clade or of the sister clade of *Atelopus* (which also includes *Osornophryne*). Finally, Type A glands are a synapomorphy of the *Rhinella granulosa* group.

Adhesive glands are the first structures to appear in all species but *Melanophryniscus atroluteus*, *M. krauczuki*, and *M. rubriventris*, where branchial arches are evident first (Table 1; Fig. 26). They reach their maximum development after the formation of the operculum at the gill base and before its medial fusion. Gland subdivision differs in bufonids relative to *Hypsiboas riojanus* and among all bufonids that we examined. It occurs much earlier in *Atelopus* and *Melanophryniscus* and generally coincides with the formation of the branchial arches or the first two gill buds. The most precocious development occurs in *A. elegans*, where glands subdivide before the tail bud is evident; the most delayed development occurs in *M. alipioi*, where glands divide after the second gill pair is formed. Conversely, in *Bufotes*, *Dendrophryniscus*, and *Rhinella* gland subdivision always occurs after the operculum is evident at the gill base, and in the most delayed cases, after its medial fusion. In *R. rumbolli* the earliest subdivision occurs slightly after the second gill pair is branched, and to a lesser extent, the three species of the *R. granulosa* group have glands that separate before the gills reach their maximum development. This agrees with the observations of Nokhbatolfoghahai and Downie (2005) about intraspecific variation in gland subdivision, which is earlier in *R. humboldti* of the *R. granulosa* group. The complete regression of adhesive glands is in general later than in *H. riojanus*, and in most cases it is the last event in the sequence after the hind limbs reach Stage 27. Regression becomes evident first with a loss of prominence of the whole structure and with strong pigmentation around the reducing

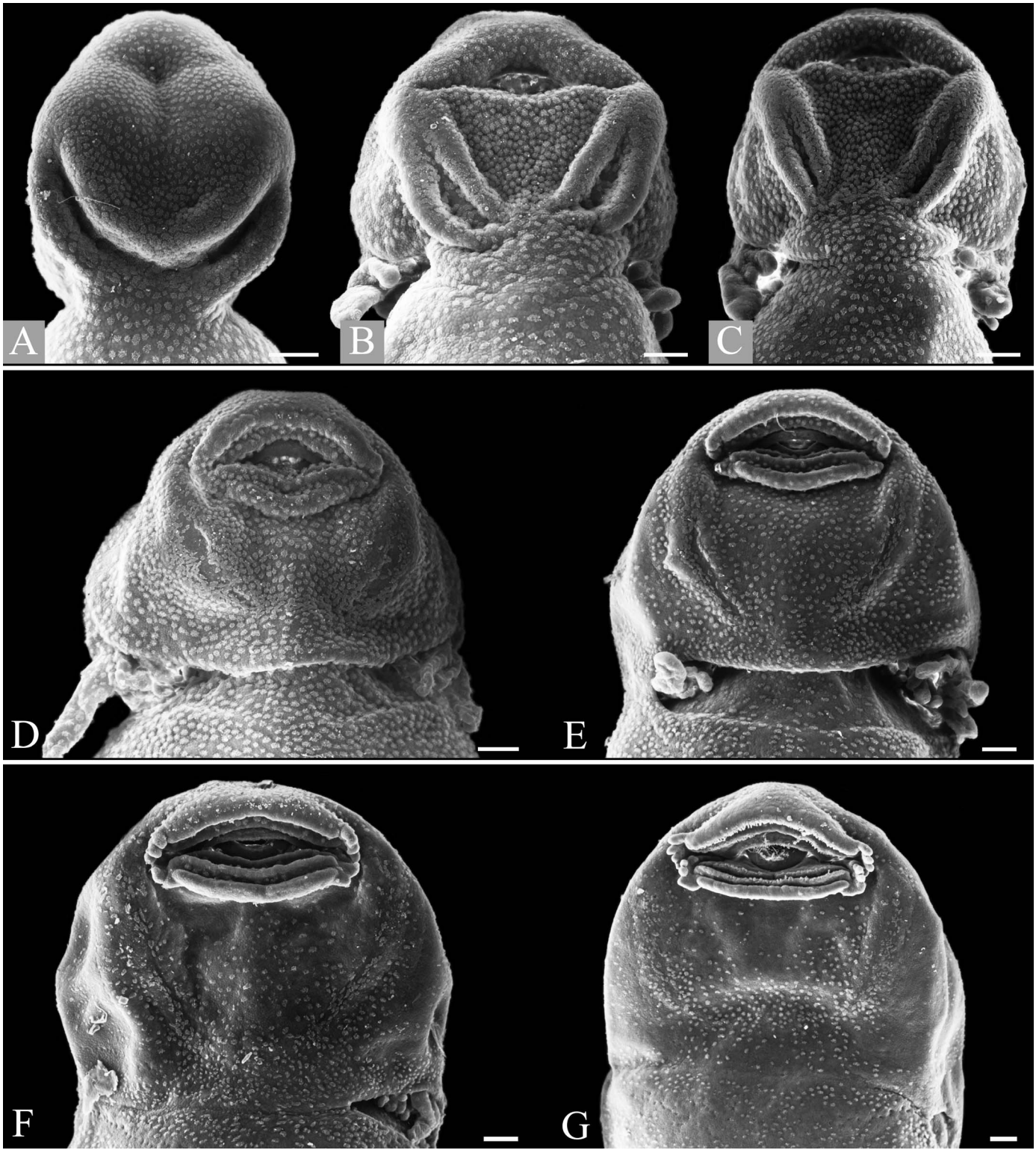


Fig. 25.—Development of *Rhinella rumbolli* without an abdominal sucker. Embryos with (A) branchial arches, (B) second pair of gills branched, (C) operculum at the gill base, (D) operculum medially fused, (E) right gill regressing, (F) left gill regressing, and (G) spiracle formed. Scale lines = 100 μm.

secretory region; small patches of secretory cells are the last vestige of the adhesive glands.

The optimization of the considered character states about gland division timing (i.e., before the operculum covers the gill bases, before the operculum fuses medially, after the operculum fuses medially) implies that the plesiomorphic condition in Bufonidae is the division before the operculum covers the bases of the gills (Fig. 27). The lack of data for *Osornophryne* does not allows an interpretation of whether the division after the operculum fuses medially is a

TABLE 1.—Ranks of developmental events used in the sequence heterochrony analyses. The differentiation of the third pair of gills (*) could not be resolved in all species and is excluded from the plots; data are designated for taxa where it was observed. The event that precedes hatching is indicated with "(h)." Ae, *Atelopus elegans*; As, *A.* aff. *spumarius*; Bv, *Bufotes viridis*; Db, *Dendrophryniscus* aff. *berthalutzae*; Hr, *Hypsiboas riojanus*, Ma, *Melanophryniscus alipioi*; Md, *M.* aff. *devincenzii*; Mk, *M. krauczuki*; Ml, *M. milanoi*; Mm, *M. macrogranulosus*; Mr, *M. rubriventris*; Ms, *M. sanmartini*; Mt, *M. atroluteus*; Ra, *Rhinella arenarum*; Rc, *R.* cf. *cerradensis*; Rf, *R. fernandezae*; Rh, *R. achalensis*; Rm, *R. major*; Ro, *R. ornata*; Rr, *R. rumbolli*; Rs, *R. spinulosa*; Rz, *R. azarai*.

	Hr	*Ae*	*As*	*Bv*	*Db*	*Ma*	*Mt*	*Md*	*Mk*	*Mm*	*Ml*	*Mr*	*Ms*	*Ra*	*Rh*	*Rz*	*Rc*	*Rf*	*Rm*	*Ro*	*Rr*	*Rs*
Adhesive gland as groove	1	1	1.5	1	6	1	2	1	3	1	2.5	3	1	1	1.5	1	1	1	1	1	1.5	1
Tail bud	2	2	1.5	3(h)	6	3	3	3	1.5	3	2.5	2	2	3(h)	3(h)	3(h)	3(h)	2	3(h)	2(h)	3(h)	3
Fin bud	3	5	3	4	6	3	4	4	5	5.5	2.5	4	3	4	4.5	4	4	3(h)	4	3	4	4
Branchial arch buds	4	4(h)	4(h)	2	6	3	1	2(h)	1.5	3(h)	2.5	1	4(h)	2	1.5	2	2	4	2	4	1.5	2
First gill pair bud	5	6	6	5	6	5	6	5	6(h)	5.5	6	6	6	5	4.5	5	5	5	5	5	5	5
Second gill pair bud	6	9	10	6	6	6	8	7	7	7.5	6	8	8	6	6	6	6	6	6	6	6.5	6(h)
First gill pair branched	7	11.5	11	7	15	7.5	7	8	8	7.5	8.5	7	9	7	7	7	7	7	7	7	8	7
Second gill pair branched	8	11.5	12	8	15	9.5	9(h)	9	11	9	10	9(h)	12	8	8.5	8	8	8	8	8	9	8
**Third gill pair bud*	*8.5*	*12.5*	*14.5*	*8.5*	*?*	*12.5*	*9.5*	*13.5*	*12.5*	*?*	*18.5*	*11*	*?*	*9.5*	*9.5*	*8.5*	*8.5*	*11.5*	*10.5*	*9.5*	*11.5*	*10.5*
Tail length/body length = 1	9	10	8	11	6	9.5	12	12	12	16.5	8.5	13	7	11	11	13	11	13	15	11	17	11
Adhesive glands separated	10	3	5	15	15	7.5	5	6	4	3	6	5	5	15	18	11	18	11	11	14	10	16
Oral disc outlined	11	7	9	9	6	11.5	11	11	10	10	18.5	10	11	9	8.5	9	10	9	9.5	10	6.5	9
Operculum at the base of gills	12	8	7	10	6	11.5	10	10	9	16.5	18.5	11	10	10	10	10	9	10	9.5	9	11	10
Gills fully developed	13	13	13	12	6	13.5	13	13	13	16.5	18.5	12	13	12	12	12	12	12	13	15	14	14
Operculum medially fused	14	14	14	13	15	16	15	16	15	16.5	18.5	14	15	13	13	15	13	15	12	13	12	12
Operculum covering right gill	15	21	16	18	19.5	20	20	19.5	19	16.5	18.5	20	17	18	19	19	20	20	20.5	20	19	19
Larval labial tooth row formula outlined	16	21	17	16	6	15	14	14.5	14	16.5	18.5	15.5	14	14	15.5	14	14	14	14	16	16	13
First teeth	17	21	22.5	19	15	18	17	17.5	16	16.5	18.5	17.5	19	20	20	18	19	18	17	19	20	20
Coiled gut	18	21	22.5	14	23.5	21	16	19.5	20	16.5	18.5	19	18	16	15.5	16	17	16	16	17.5	14	17
Operculum covering left gill	19	21	18	21	21.5	22(h)	21	21	21	16.5	18.5	24	20	21	21	21	22	22	23	21	21	21
Marginal papillae complete	20	21	22.5	20	15	18	19	17.5	17	16.5	18.5	17.5	21	19	15.5	17	16	19	18.5	17.5	18	15
Oral disc fully developed	21	21	22.5	23	21.5	23.5	22	22.5	22	16.5	18.5	24	24	22	24	22	21	23	22	24.5	24	23
Limb bud	22	15	15	17	15	13.5	18	14.5	18	16.5	18.5	15.5	16	17	15.5	20	15	17	18.5	12	14	18
Spiracle	23	21	22.5	25	23.5	23.5	23	22.5	23	23.5	18.5	24	23	24	24	24	24	24	25	24.5	22	25
Adhesive glands not visible	24	21	22.5	24	25.5	25.5	26	26	26	26	18.5	21	26	26	24	26	26	26	26	26	25.5	24
Limb at Gosner Stage 27	25	21	22.5	26	19.5	18	25	24.5	25	23.5	18.5	24	25	25	24	25	25	25	24	22	25.5	26
Active feeding	26	21	22.5	22	25.5	25.5	24	24.5	24	25	18.5	24	22	23	24	23	23	21	20.5	23	23	22

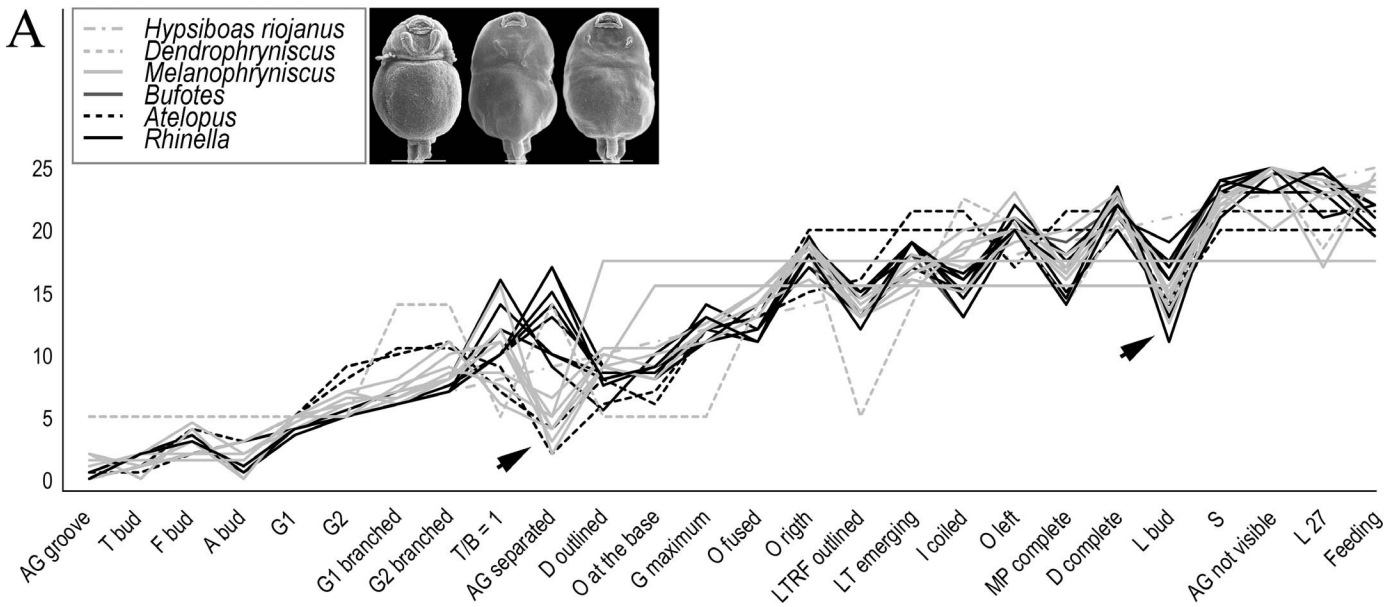


FIG. 26.—Sequence heterochrony in early ontogeny of bufonids as plotted from data in Table 1. The X axis displays the developmental events ordered as they occur in the reference trajectory, and the Y axis shows the ranks (i.e., order of occurrence) for each event. Developmental events scored in 21 species as compared to the reference trajectory of *Hypsiboas riojanus*. Lines with plateaus correspond to incomplete series. A = branchial arches, AG = adhesive glands, B = body, D = oral disc, F = fin, G = gill, I = intestine, L = limb, LT = labial tooth, MP = marginal papillae, O = operculum, T = tail. Note the diversity in the time that the adhesive glands divide and the early differentiation of hind limbs in all bufonids (arrows). The inset photograph shows the hind limb buds co-occurring with the external gills in *Dendrophryniscus*. aff. *berthalutzae*.

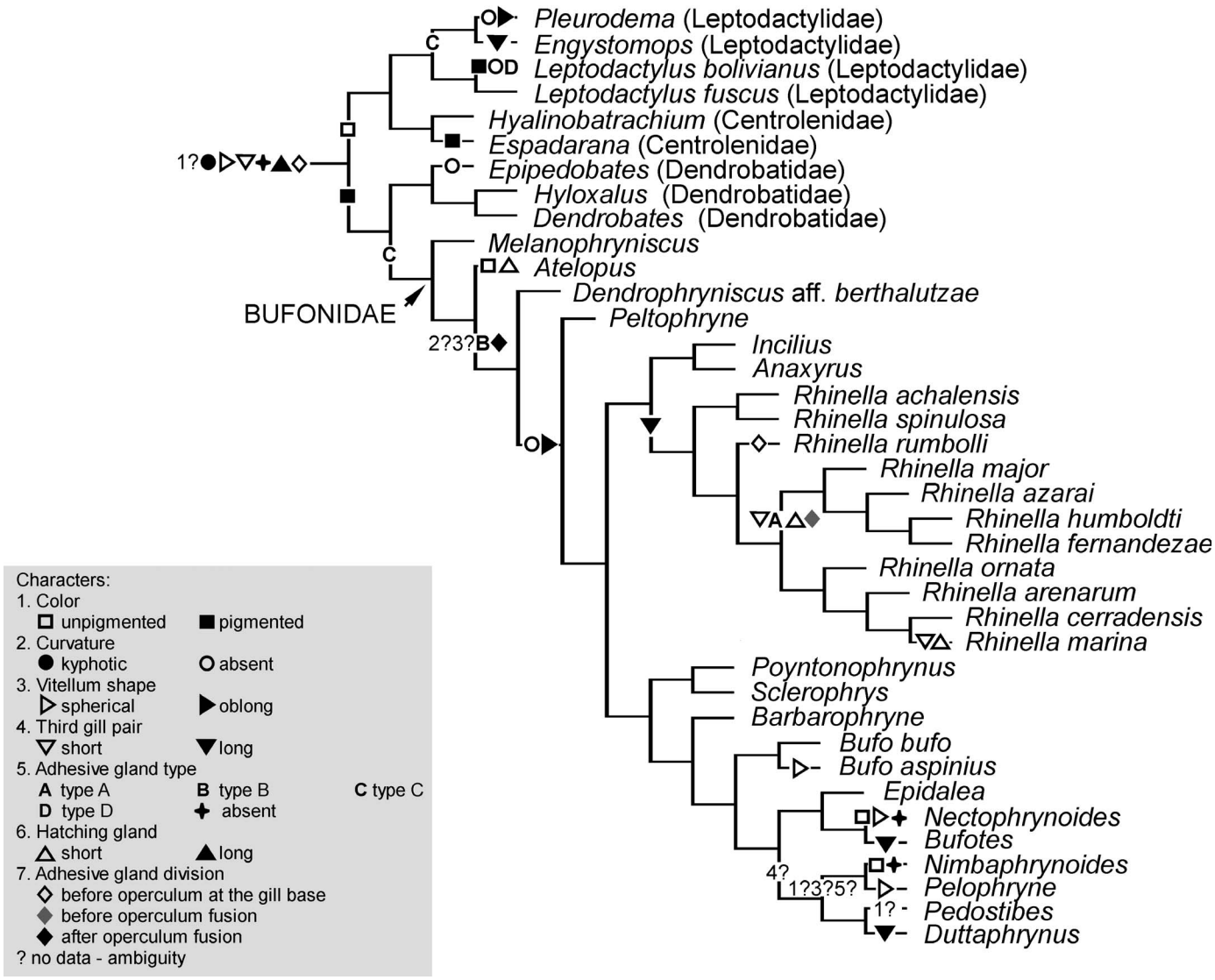


FIG. 27.—Ancestral state reconstructions for the evolution of morphological and developmental characters on the phylogenetic hypothesis of Pyron (2014) based on the data of the Appendix. We did not include taxa without information, and we added *R. granulosa* and *R. marina* groups from data in Pereyra et al. (2016) and Maciel et al. (2010), respectively (see also Pramuk et al. 2008; van Bocxlaer et al. 2010; Ron et al. 2015). Characters and states are defined as follows: (1) color at tail-bud stage, unpigmented, pigmented; (2) dorsal curvature at tail-bud stage, kyphotic, absent; (3) yolk shape, spherical, oblong; (4) size of the third pair of gills, short—not extending beyond the operculum margin—or absent, and long—extending beyond the operculum margin; (5) adhesive gland type, A, B, C, D, glands absent; (6) dorsal line of hatching gland, short—restricted to the cephalic region, long—extending beyond the head; (7) timing of the division of the adhesive gland, before the operculum covers the bases of the gills, before the operculum fuses medially, after the operculum fuses medially. Note the early appearance of the widespread Type B adhesive glands within the Bufonidae and the synapomorphies defining the *R. granulosa* group.

synapomorphy of the sister clade of *Atelopus* or of *Osornophryne*. The division before the operculum fuses medially is a synapomorphy of *Rhinella granulosa* group, and the reversion to the plesiomorphic condition in *R. rumbolli* is an autapomorphy of this species, which also shows that correlation between gland type and division timing does not always occur.

Hatching Gland
(Figs. 13–16)

In all embryos, except the unpigmented *Atelopus* (Fig. 13A,B), the hatching glands are in a pigmented region on the anterodosal part of the head after the tailbud stage (Fig. 13C–U). The frontal region has a T- or Y-shaped field that is easily visible in SEM analysis as a nonciliated area. The dorsal line of this field reaches various distances. In species of the *Rhinella granulosa* group (Fig. 13O–Q), it barely extends beyond the posterior limit of the head, whereas in all the remaining *Rhinella* it may extend to half the body length (e.g., Fig. 13M,N). In *Bufotes* (Fig. 13C), the pattern is similar to these *Rhinella* species. In *Dendrophryniscus*, the gland at Stage 23 (we lacked earlier specimens) extends to the posterior edge of the head (Fig. 13D). In *Melanophryniscus* embryos (Fig. 13E–L), the intensely pigmented dorsal line is particularly long and may reach the base of the dorsal fin in *M. alipioi* and *M. milanoi* (Fig. 13G,H). In *M. atroluteus* and *M. rubriventris* the line of glands is short (Fig. 13K,L). In several cases (e.g., *R.*

fernandezae; Fig. 13O) the line shifts to one side or the other, especially in the frontal region. Hatching gland cells are arranged uniformly or are arranged as small, aligned groups of 2–4 cells (Figs. 14 and 15). Most of the species have the continuous pattern, and isolated groups can be seen in *M. rubriventris*, *R. achalensis*, *R. azarai*, *R. fernandezae*, and *R. rumbolli* (Figs. 14C,E and 15C). Individual hatching cells vary in size, shape, and the length and density of microvilli. Microvilli are longer in *Melanophryniscus* than in other genera (large patches of hatching cells in *M.* aff. *devincenzii* and *M. krauczuki* resemble ciliated cells; Fig. 15D,E), and interspecific variations include less dense microvilli in *M. atroluteus*, *M. rubriventris*, and *M. sanmartini* (Fig. 15A–C,F). In *Atelopus* (Fig. 16A), the structure is evident only with SEM observations, which show hatching gland cells isolated or in small groups concentrated on the frontal region; microvilli in these species are comparatively short. Micrographs of *Bufotes viridis* show a narrow line with small hatching gland cells with short microvilli (Fig. 16B).

Some of our results are similar to previous reports in bufonids (Nokhbatolfoghahai and Downie 2007). *Rhinella humboldti* embryos show a dorsal, discontinuous line like *R. azarai* and *R. fernandezae* of the same intrageneric group. Conversely, the distribution restricted to the frontal region in *R. marina* embryos differs from all other species in our sample. The ancestral state reconstruction indicates the dorsal line that extends over most of the head region is the plesiomorphic condition in Bufonidae, whereas a short dorsal line mostly restricted to the cephalic region is a synapomorphy of *Atelopus* and the *R. granulosa* group with an instance of homoplasy in *R. marina* (see Fig. 27).

Events of hatching gland development were not included in the sequence heterochrony analysis because (1) we lack SEM pictures of late stages of all species to assess when hatching gland cells are no longer present, and (2) images made with a stereomicroscope that show gland presence based on the pigmentation of the frontal and dorsal lines are not suitable to assess gland presence in unpigmented *Atelopus*. Nevertheless, we could recognize some changes in the stage of regression of the hatching gland in most species based on pigmentation. In some *Rhinella* species, such as *R. arenarum* and *R. ornata*, the dorsal line is evident until the spiracle develops (Stage 25). In *R.* cf. *cerradensis* and *R. rumbolli* the gland is no longer visible when the operculum covers the right gill. The earliest regression in the genus appears to be that in the *R. granulosa* species groups where the dorsal line is not visible around Stage 23 shortly after the adhesive glands split. The glands of *Bufotes viridis* and *Dendrophryniscus* aff. *berthalutzae* follow a similar pattern as in most *Rhinella*, with glands not evident around Stage 24. In most *Melanophryniscus* regression occurs around Stage 24. The most persisting glands occur in *M. alipioi*, where a darkly pigmented dorsal line is seen until hind limbs reach Stage 27. Likewise, in *M. krauczuki* hatching glands occur until the hind limbs are at Stage 26. Developmental patterns of the hatching glands, including stages of regression, are similar in previously described bufonids (Nokhbatolfoghahai and Downie 2007).

Body Ciliation
(Figs. 17–18)

Structural variations among cilia patterns of bufonids are seen in general density, distribution across body regions, and size and shape of individual cells. Ciliation patterns change ontogenetically (Fig. 17), and after an interspecifically variable stage of maximum ciliation, cilia density is reduced in specific areas until cells disappear completely. Ciliation is more profuse in *Bufotes*, *Dendrophryniscus*, and *Rhinella* than in *Atelopus* species (see Figs. 5 and 24). Within *Rhinella*, species of the *R. granulosa* group have comparatively fewer cilia, and *R.* cf. *cerradensis* and *R. ornata* have the densest arrangement of cilia. Species of *Melanophryniscus* are less ciliated, and among them, *M. atroluteus* has the highest density, and *M. alipioi*, *M.* aff. *devincenzii*, *M. krauczuki*, and *M. macrogranulosus* have few ciliated cells (e.g., Figs. 6 and 7).

In *Bufotes*, *Dendrophryniscus*, and *Rhinella* with Type A and B adhesive glands, the oral and gular regions are densely ciliated. Glands and the area between them show large cells with very long cilia, and in younger embryos of *R. ornata*, the entire region appears covered by continuous patches of ciliated cells (Fig. 5A,B). In *R. rumbolli*, ciliated cells occur on the secretory region of the glands, scattered among cells with microvilli (Fig. 25B). Conversely, in *Melanophryniscus* species the Type C glands are scarcely ciliated at early stages of development (Figs. 6, 7, and 11). External gills also vary in their degree of ciliation, from *R. ornata* with densely ciliated gills (Fig. 5) to *M. alipioi* and *Atelopus* with a few scattered ciliated cells on the main gill branches.

Most species have rounded or polygonal ciliated cells that are typically larger than the nonciliated epidermal cells (Fig. 18). Ciliated cells tend to become larger and with longer cilia during ontogeny, except in *Melanophryniscus krauczuki* embryos, which have relatively small cells throughout development (Fig. 18E). *Dendrophryniscus*, *Rhinella achalensis*, and *R.* cf. *cerradensis* have longer and narrower ciliated cells with the size of 2–6 epidermic cells generally arranged transversely to the body longitudinal axis (Figs. 17 and 18C). We found no clear pattern in the density, size, and shape of ciliated cells that might be correlated with ecological aspects of oviposition mode and tadpole development.

Ontogenetic changes in body ciliation could not be included on the sequence heterochrony analyses because we lacked SEM images of some species and stages. The primary changes concern the onset of ciliation regression and the stage at which cilia are mostly absent. Cilia begin to regress between Stages 23 and 25 and the latest stages for regression occur in *Rhinella* cf. *cerradensis* and *R. ornata*. By Stage 26–27, ciliation is almost gone in most species, except for the lateral and gular regions of the body that are usually the last to loose ciliated cells. The earliest stage corresponds to embryos of *Melanophryniscus* aff. *devincenzii*, which almost lack body ciliation by Stage 25. On the other extreme, embryos of *Dendrophryniscus* aff. *berthalutzae* and *M. alipioi* have hind limbs at Stage 28–29 and still have dense cilia.

Oral Disc
(Figs. 19–23 and 26)

Most species in our study share an oral disc with labial tooth row formula (LTRF) of 2/3 and marginal papillae with

dorsal and ventral gaps (Figs. 19–21). The ventral gap is a synapomorphy of Bufonidae (Haas 2003; Frost et al. 2006) that is secondarily absent in several species of this family (e.g., *Ansonia*, Haas et al. 2009; *Leptophryne*, Berry 1972; most species of *Mertensophryne*, Channing et al. 2012; *Nimbaphrynoides occidentalis*, Lamotte and Xavier 1972a; *Rhinella scitula*, Caramaschi and Niemeyer 2003; *Sabahphrynus maculatus*, Inger 1992; *Werneria*, Channing et al. 2012). Formation of this gap occurs simply because the development of the marginal papillae, that first appear laterally and progress medially, is interrupted. This pattern results in ventral gaps of various lengths (73.8–92.3% of the oral disc width). In turn, labial ridges develop in a similar way in all embryos. Labial ridges A1 and P1 are the first to differentiate, and the remaining rows appear almost simultaneously slightly later, both in those species with LTRF 2/2 (*Rhinella granulosa* group; Fig. 20A–C) and 2/3 (the rest; Figs. 20D–F and 21). These observations indicate that, unlike in other groups (e.g., *Physalaemus*, Vera Candioti et al. 2011), a 2/2 oral disc does not result from a truncated development of a 2/3 one, because the latter taxa never go through a 2/2 stage. Row A2 can be complete (e.g., *Atelopus*, *Dendrophryniscus*, all *Melanophryniscus*, and *R. rumbolli*) or divided (*Bufotes*, and the remaining *Rhinella*), and in all cases labial teeth first emerge at the central region of each half-ridge and then progress laterally to complete the row. In most species the complete (e.g., most *Melanophryniscus*, and *R. granulosa* group) or divided (e.g., *Bufotes*, and all other *Rhinella* but *R. rumbolli*) row P1 is outlined as an indented ridge and teeth appear first at the central region of each section. Conversely, in *R. rumbolli*, this ridge appears early as a transverse, not indented prominence, although teeth follow the same developmental pattern and an incipient division is seen in older stages in some specimens. In *Atelopus,* the P1 row appears complete but data about tooth emergence could not be recorded. When present, P3 is invariably the last row to form teeth at about Stage 25. Individual labial teeth (Figs. 22 and 23) vary in size (e.g., teeth are smaller and shorter in *M. alipioi*; Fig. 22B) and cusp pattern (e.g., few distal cusps in species with LTRF 2/2; Fig. 23D,E; Vera Candioti and Altig 2010; Baldo et al. 2014).

In most species, the oral disc begins to widen and the labia differentiate once the first two gill pairs are branched (Table 1; Fig. 26). In *Atelopus elegans* and *Rhinella rumbolli* this occurs while the gills develop. The definitive larval LTRF is acquired almost simultaneously across genera, whether the oral disc has two or three lower tooth ridges. Except for *Atelopus*, this always precedes the regression of the right gill. Teeth usually emerge earlier in the developmental sequence of *Melanophryniscus* than in *Rhinella* but always before complete development of the marginal papillae. In all species but *Dendrophryniscus* and *M. alipioi*, when it occurs earlier, a completely developed oral disc is acquired shortly before the hind limbs reach Stage 27. Within *Melanophryniscus*, embryos of lotic species with the proportionately largest larval discs (i.e., *M.* aff. *devincenzii*, *M. krauczuki*, and *M. macrogranulosus*; >30% of the body length; see also Baldo et al. 2014) complete their configurations earlier than the remaining species (Table 1; Fig. 26). Within *Rhinella*, species with LTRF 2/2 (*R. granulosa* group) do not complete their discs before those with LTRF 2/3, which supports the idea that the 2/2 configuration does not represent a truncated development (Table 1; Fig. 26). Unfortunately, we have no data about when the largest discs of *Atelopus* reach their larval shape. In comparison, *A. flavescens* with a well-developed spiracle have well-defined marginal papillae and labial tooth row formula (Gawor et al. 2012). Other bufonids, such as *Anaxyrus* and *Incilius*, show a comparable developmental sequence of mouth parts (e.g., tooth ridges, labial teeth, marginal papillae; Limbaugh and Volpe 1957; Tubbs et al. 1993).

In some species, the development of the oral disc shows some correlation with that of the gut and the beginning of the active feeding. In most species, the gut develops its first coils well before the oral disc is completed (Table 1; Fig. 26). *Bufotes viridis* and *Rhinella rumbolli* develop the gut coils quite early. Species of the *R. granulosa* group slightly precede other *Rhinella*. Among the latest, *Atelopus* and *Melanophryniscus alipioi* are followed by *M.* aff. *devincenzii* and *M. krauczuki*. In *Dendrophryniscus* aff. *berthalutzae*, oral disc configuration is acquired before the gut coils develop. Active feeding begins approximately with the defined oral disc, slightly early in *Rhinella* and slightly later in *Melanophryniscus*. Feeding starts much later in *Dendrophryniscus* and *M. alipioi*.

Abdominal Sucker
(Figs. 24 and 25)

Within Bufonidae, larvae of *Atelopus*, *Sabahphrynus maculatus*, and some species of *Bufo* and of the *Rhinella veraguensis* group have an abdominal sucker (Cadle and Altig 1991; Inger 1992; Rao and Yang 1994; Aguayo et al. 2009). This structure is a modification of the abdominal skin with a bulbous rim delimiting a central, depressed area with changes in several muscles and skeletal parts underlying the integument (F. Vera Candioti, personal observation). The embryological origin of the abdominal sucker is not clear. Based on observations of *B. aspinius*, Rao and Yang (1994) suggested that the sucker develops from the adhesive glands. Our observations in *Atelopus* embryos show that the sucker rim appears around the adhesive glands and has an independent development (Fig. 24). It begins to form at Stage 24, well after the right gill is concealed by the operculum. Lateral margins are first evident as longitudinal ridges that delimit a slightly prominent border (Fig. 24C). Unfortunately, we lack older specimens, so we are not certain when the sucker is completely developed. At about Stage 25, the posterior, transverse section of the rim is not yet developed (Fig. 24D). As the sucker becomes more prominent, the adhesive glands (which in *Bufo* are Type B) regress, and this could be the source of confusion in Rao and Yang (1994). Our observations also differ from previous descriptions of early embryos of *Atelopus* spp., where the abdominal sucker is reported to develop from Stage 21 (Mebs 1980; Karraker et al. 2006; Gawor et al. 2012). Although heterochronic shifts could occur in the onset of the sucker differentiation, there is no mention of adhesive glands in those descriptions, and we suspect that the large adhesive glands is what were previously described as the abdominal sucker. The rather late development of the abdominal sucker implies that they would not be responsible for the adhesion of young (about Stage 23) embryos of *A. cruciger* described by Mebs (1980). Instead, adhesive glands at this point are still huge, with a large area of secretory cells that are likely

the main adhesion organ in these embryos. They are likely not as efficient such as the eventual sucker, as Karraker et al. (2006) mentioned that newly hatched *A. zeteki* embryos cannot adhere to inverted rocks. The switch from the adhesive glands to the sucker as the main adhesion function is an interesting transition that should be compared interspecifically.

Finally, we included an embryonic series of the rheophilous *Rhinella rumbolli*, a species belonging to the *R. veraguensis* group that lacks the abdominal sucker distinctive of an internal clade of the group (Fig. 25; Pereyra et al. 2015). The areas lateral to the gular area, which are characteristic of these larvae (Haad et al. 2014), are obvious from a late Stage 23 and are occupied mainly by each half of the B-type adhesive gland. There is no evidence of heterochronic change, such as an initial morphogenesis of the lateral sections of the rim of the sucker.

Hind Limb Differentiation (Fig. 26)

Hind-limb differentiation defines Gosner Stage 26, and it is almost universally considered the beginning of the larval period. In several species, it follows the formation of the spiracle, so hind limbs do not usually co-occur with external gills. Among the species we examined, hind limbs were always precocious relative to other morphological features and occurred even before the operculum covers the right gill. In general terms, it is earliest in most *Melanophryniscus*, *Atelopus* spp., and *Dendrophryniscus* aff. *berthalutzae*. Embryos of *Bufo bufo* and *D. brevipollicatus* also develop their hind limbs when gills are still exposed (Khan 1965; Izecksohn and da Cruz 1972). In the earliest examples, such as in *M. alipioi* and in *Rhinella ornata*, limb buds are evident before the operculum fuses medially. Limb bud development is slightly later in *Bufotes*, *M. krauczuki*, *R. arenarum*, *R. spinulosa*, and species of the *R. granulosa* group (Table 1). Once present, the hind-limb buds develop at a similar rate in all species except for *D.* aff. *berthalutzae* and *M. alipioi*, where the limbs reach Stage 27 before the operculum covers the left gill.

Tail Development (Fig. 26)

The development of the tail is highly variable. Although the tail and fin bud differentiation are the first events to occur during the ontogeny, the rate of growth of the tail differs interspecifically. The tails of *Atelopus* species, *Melanophryniscus alipioi*, and *M. sanmartini* equal the body length earlier than in the remaining species. Within *Rhinella*, *R. spinulosa* is the earliest and *R. rumbolli* the latest to acquire a 1:1 tail–body ratio (Table 1).

Hatching

Our observations show that hatching stage is highly stable within and between ovipositions of the same species (Table 1), but wider samples including several ovipositions from different environments across the whole distribution should be examined to confirm it. Timing of hatching is quite variable interspecifically. In general, bufonids hatch early at about the tail-bud stage (del Conte and Sirlin 1952; Limbaugh and Volpe 1957). Accordingly, *Bufotes* and most *Rhinella* embryos hatch at tail-bud stage before the caudal fin is formed. *Rhinella spinulosa* embryos hatch after the first two pairs of gills are differentiated. Hatching of *Melanophryniscus* is slightly later and more variable interspecifically. The species that hatch the earliest are *M. sanmartini* and both species of the *M. tumifrons* group, which hatch before the first pair of gills emerges. *Melanophryniscus krauczuki* embryos hatch slightly later, before the differentiation of the second gill pair. Species of the *M. stelzneri* group hatch after the second pair of gills is branched. And finally, *M. alipioi* embryos vary in hatching time and can be as late as when the operculum covers the left gill. Likewise, *Amazophrynella minuta* embryos are capable of remaining intracapsular for a long period (Thibaudeau and Altig 1999). *Atelopus* embryos hatch before the gills start to develop, at about Stage 19–20 (Mebs 1980; Gawor et al. 2012; this work).

Synthesis

The comparative analyses of early ontogenetic trajectories of bufonids revealed interspecific variations in morphological and developmental patterns of embryonary and larval features. In particular, the approach of sequence heterochrony allowed us to compare developmental trajectories in species from diverse geographic regions and environments where absolute time data would be not comparable. With this method, both general ontogenetic patterns and subtle temporal shifts became evident. The occurrence of the earliest events (i.e., those referring to tail and external gills differentiation) is in general highly conserved among bufonids and relative to the hylid trajectory. Later events show several degrees of interspecific variations. The primary changes within *Rhinella* include the onset of the split of adhesive glands and the rate of tail lengthening, whereas the stage of hatching and the rate of tail and hind limb development are the primary shifts in *Melanophryniscus*. Additionally, associations between the occurrences of some events are maintained. The development of the labial tooth row formula before gill regression and the emergence of the hind-limb buds before the oral disc is complete are conserved among bufonids but differ from hylid trajectories (F. Vera Candioti, personal observation). Provided that a set of events are moving together along a trajectory, these comparisons can be helpful to identify patterns of developmental integration and modularity and their diversity in different lineages (Smith 2001).

Morphological and developmental aspects of the early ontogeny have proven useful in the definition of clades in Bufonidae, and character evolution can be explored in comprehensive phylogenetic contexts (Fig. 27). The reconstruction of ancestral states suggests several putative synapomorphies for the family and identifies some traits consistent with genera diversification and features exclusive of internal clades. As with larval and adult characters (e.g., Haas 2003), embryos of *Melanophryniscus* and *Atelopus* differ widely from those of other bufonid clades (e.g., body curvature and adhesive gland type). In more derived groups some features remain conserved (e.g., Type B adhesive gland that splits later in development) and often change in particular taxa in relation to ecomorphological aspects (e.g., species with endotrophic development and glands absent). Within *Rhinella*, the *R. granulosa* group is defined by four synapomorphies (Type A adhesive glands that divide early, a short dorsal hatching gland, and a poorly developed third

pair of gills). Pereyra et al. (2016) proposed a labial tooth row formula with two lower tooth ridges as one putative synapomorphy of the *R. granulosa* group, with reversions to three lower ridges in a derived clade including *R. humboldti*. This is interesting because this species, and probably the other four in this derived clade, have a LTRF 2/3 that is ancestral for *Rhinella* but maintains the diagnostic embryonic features of the *R. granulosa* group. Within *Melanophryniscus*, comparisons between developmental patterns across intrageneric groups require a more complete sampling.

Bufonids exhibit a wide variety of oviposition sites and types, developmental modes, and numerous combinations of morphological and ecological aspects of embryos and tadpoles (e.g., van Bocxlaer et al. 2010). Among the sample we studied, at least three general larval ecomorphological guilds are represented. Tadpoles of *Atelopus*, *Melanophryniscus krauczuki*, species of the *M. tumifrons* group, *Rhinella rumbolli*, and to a lesser extent *R. ornata* inhabit flowing water (e.g., Lötters 2001; Baldo et al. 2014; Pereyra et al. 2015). Embryos of *Dendrophryniscus* aff. *berthalutzae*, *M. alipioi*, and *M. milanoi* develop in the water accumulated in leaves of terrestrial or epiphytic plants of various families (Izecksohn 1993; Bornschein et al. 2015). The remaining species have pond-type larvae inhabiting various temporary or permanent bodies of lentic water (e.g., Sicilia et al. 2006; Tolledo and Toledo 2010; Kolenc et al. 2013; Baldo et al. 2014; Blotto et al. 2014; Haad et al. 2014). Some of the morphological and developmental variations found could be related to the environments where embryos and tadpoles develop.

Tadpoles of lotic water bodies often exhibit features related to resist flowing water. Large oral discs, for example, appear to be convergent in several lineages of anuran larvae (e.g., Altig and McDiarmid 1999). Furthermore, Nodzenski and Inger (1990) found that oral structures of suctorial larvae of *Ansonia* (Bufonidae) and gastromyzophorous tadpoles of *Meristogenys* (Ranidae, and in this case also the abdominal sucker) metamorphose later relative to other developmental events, so the functional duration of those structures is extended. In our case, embryos of *Atelopus*, *Melanophryniscus* aff. *devincenzii*, *M. krauczuki*, and *M. macrogranulosus* have the largest adhesive glands and oral discs, and in *Melanophryniscus*, the development of the oral discs is completed earlier than in the other species. Lotic species of *Melanophryniscus* are also among the earliest to hatch, so the large adhesive glands and developing oral disc are likely functional early. There are no clear ecomorphological patterns in embryonic ontogeny of *Rhinella rumbolli* and *R. ornata* linked to the water bodies where they develop.

Tadpoles that develop in phytotelmons have evolved in several anuran clades, and ecomorphs associated to these microhabitats are highly variable (Lehtinen et al. 2004). Early developmental patterns are scarcely known in these species, but some features are shared among our small sample. Like in several species with terrestrial oviposition, such as centrolenids, phyllomedusines, and batrachylids (e.g., Salica et al. 2011; Nokhbatolfoghahai et al. 2015; J. Grosso, personal observation), hatching is late in *Dendrophryniscus* and *Melanophryniscus alipioi*. The yolk mass is large and persistent in these embryos, and this condition delays the development of the gut and the onset of active feeding. Although the early development of the hind limbs is common to all bufonid embryos that we studied, disregarding variations in oviposition sites and developmental modes, phytotelmon embryos have an increased rate of the hind limb differentiation so that large limbs co-occur with body ciliation, a recently formed spiracle, and adhesive gland remnants (see Fig. 26). Likewise, arboreal larvae of the bufonid *Pedostibes tuberculosus* show remnants of adhesive glands and yolk at Stage 27 (Dinesh and Radhakrishnan 2013). Some of the traits of phytotelmon embryos in this study are frequent in other species with terrestrial development (*Batrachyla*, petropedetids; Drewes et al. 1989; J. Grosso, personal observation), and in endotrophic tadpoles (e.g., *Allobates nidicola*, *Altiphrynoides malcomi*, *Nectophryne*; Wake 1980; Caldwell and Lima 2003; Channing et al. 2012). Endotrophy occurs within phytotelmons in several anuran lineages (e.g., *Blythophryne beryet*, *Frostius*, *Flectonotus*, *Pelophryne*, and *Syncope*; Cruz and Peixoto 1982; Duellman and Gray 1983; Krügel and Richter 1995; Leong and Teo 2009; Chandramouli et al. 2016). In *Dendrophryniscus*, Izecksohn and da Cruz (1972), while correcting tadpole identification by de Carvalho (1949), noted that the yolk of *D. brevipollicatus* persists until metamorphosis, and thus larvae likely do not feed during development. Premetamorphic tadpoles of *D.* aff. *berthalutzae* in our study (hind limbs about Stage 34) have a large amount of yolk within the digestive tract along with macrophyte detritus. Similar observations recorded in phytotelm-dwelling tadpoles of *Melanophryniscus* (Baldo et al. 2014) indicate that the beginning of active feeding is delayed or that endotrophy is facultative, as known in other toads (*Incilius periglenes* and *Rhaebo haematiticus*; Crump 1989; McDiarmid and Altig 1990).

In summary, comparative analysis of ontogenetic trajectories shows wide variations in bufonid embryonic and larval features at structural and heterochronic levels. The real variation is clearly not well understood because the early development of less than 10% of the known diversity of Bufonidae (Frost 2016) has been studied. Additional information on embryonic development on early diverging genera of Bufonidae (i.e., *Amazophrynella*, *Frostius*, *Nannophryne*, *Osornophryne*, and *Oreophrynella*) will allow us to verify some patterns that we identified and establish correlations between different modes of reproduction and anatomy of embryos in this diverse group of toads.

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Appendix

Material examined in this study (*) and species with information on embryos in the literature. Seven embryonic features were used in the ancestral state reconstruction. States not applicable or with no data are designated with a question mark. The development of the hatching gland and the moment of adhesive gland division (which we assessed on scanning electron microscopy pictures or through a close examination of fresh material) were assigned with a question mark in bibliographic data. Sizes of embryos, tadpoles, and adults used in Fig. 3 are designated for the species that we examined (average, standard deviation, or range).

	Collection number	Site	Clutch type
Anaxyrus americanus			String
Atelopus cruciger			String
**Atelopus elegans*	QCAZ-A 58417	Captive. Durango, Esmeraldas, Ecuador	String
Atelopus flavescens			String
**Atelopus* aff. *spumarius*	QCAZ-A 58418	Captive. Limón, Morona Santiago, Ecuador	String
Barbarophryne brongersmai			?
Bufo aspinius			String
Bufo bufo			String
Bufo japonicus			String
Bufo torrenticola			String
**Bufotes viridis*	LGE 14000	Oltina, Constanta, Romania	String
**Dendrophryniscus* aff. *berthalutzae*	DZUP 348	Serra da Graciosa, PR, Brazil	Clump
Dendrophryniscus brevipollicatus			Clump
Dendrophryniscus leucomystax			Clump
Duttaphrynus melanostictus			String
Epidalea calamita			String
Incilius valliceps			String
**Melanophryniscus alipioi*	DZUP 351	Serra do Capivari, PR, Brazil	Isolated eggs
**Melanophryniscus atroluteus*	LGE 02644, 03842–3	Ñu Pyahú, Misiones, Argentina	Mass
**Melanophryniscus* aff. *devincenzii*	LGE 02791; 10187	Ñu Pyahú, Misiones, Argentina	Mass
Melanophryniscus klappenbachi			Mass
**Melanophryniscus krauczuki*	LGE 10180, 10184, 10193, 10195	Ñu Pyahú, Misiones, Argentina	Mass
**Melanophryniscus macrogranulosus*	MCP 8470	Morro da Gruta, RGS, Brazil	Mass
**Melanophryniscus milanoi*	MNRJ 90343	Parque Estadual da Serra do Tabuleiro, SC, Brazil	Isolated eggs
**Melanophryniscus rubriventris*	LGE 10192; FML 29227	Reyes, Jujuy, Argentina ; Abra de Cañas, Jujuy, Argentina	Mass
**Melanophryniscus sanmartini*	LGE 10467	Penitente, Lavalleja, Uraguay	Mass
Nectophrynoides tornieri			?
Nimbaphrynoides occidentalis			?
Pedostibes tuberculosus			Film/open clump
Pelophryne brevipes			?
Peltophryne taladai			Mass
Poyntonophrynus dombensis			String
**Rhinella achalensis*	LGE 04235, 10924	Pampa de Achala, Córdoba, Argentina	String
**Rhinella arenarum*	FML 29228	El Siambón, Tucumán, Argentina	String
**Rhinella azarai*	LGE 05842	Garupá, Misiones, Argentina	String
**Rhinella* cf. *cerradensis*	LGE 06338	Virasoro, Corrientes, Argentina	String
**Rhinella fernandezae*	LGE 10183, 07008	Cerro, Montevideo, Uraguay; Ituzaingó, Corrientes, Argentina	String
Rhinella humboldti			String
**Rhinella major*	LGE 07977	Suncho Corral, Santiago del Estero, Argentina	String
Rhinella marina			String
**Rhinella ornata*	LGE 00140	Salto Encantado, Misiones, Argentina	String
**Rhinella rumbolli*	LGE 06457–8	Ocloyas, Jujuy, Argentina	Clump
**Rhinella spinulosa spinulosa*	LGE 09802	Iruya, salta, Argentina	String
Sclerophrys arabica			String
Sclerophrys regularis			String
Outgroup Leptodactyliformes			
Engystomops pustulosus			
Pleurodema brachyops			
Leptodactylus bolivianus			
Leptodactylus fuscus			
Dendrobates auratus			
Epipedobates machalilla			
Hyloxalus vertebralis			
Hyalinobatrachium fleischmanni			
Hyalinobatrachium orientale			
Espadarana callistoma			

[1] Egg size estimated from the egg size in *Dendrophryniscus brevipollicatus*.

APPENDIX
Extended.

Dorsal curvature	Yolk shape	Pigmentation	Third gill pair	Hatching gland	Adhesive gland type	Adhesive gland division	Embryo length (mm)	Tadpole SVL (mm)
Straight	Oblong	Pigmented	Short or absent	?	B	?		
Kyphotic	Spherical	Unpigmented	?	?	?	?		
Kyphotic	Spherical	Unpigmented	Short or absent	Short	C	Before operculum at gill base		
Kyphotic	Spherical	Unpigmented	Short or absent	?	?	?		
Kyphotic	Spherical	Unpigmented	Short or absent	Short	C	Before operculum at gill base	2.24 ± 0.03	7.5
?	Oblong	Pigmented	Long	?	B	?		
?	Spherical	Pigmented	Short or absent	?	B	?		
Straight	Oblong	Pigmented	Short or absent	Long	B	After operculum fused		
?	?	?	?	?	B	After operculum fused		
?	?	?	?	?	B	After operculum fused		
Straight	Oblong	Pigmented	Long	Long	B	After operculum fused	2.18 ± 0.11	12.16 ± 3.99
?	?	Pigmented	?	Long	B	After operculum fused	3[1]	4 (3.8–4.2)
?	?	Unpigmented	?	?	?	?		
?	?	Pigmented	?	?	?	?		
Straight	Oblong	Pigmented	Long	?	B	?		
Straight	Oblong	Pigmented	Short or absent	?	B	?		
Straight	Oblong	Pigmented	Short or absent	?	B	?		
Kyphotic	Spherical	Pigmented	Short or absent	Long	C	Before operculum at gill base	3.5	5.7 (5.5–6.1)
Kyphotic	Spherical	Pigmented	Short or absent	Long	C	Before operculum at gill base	2.38 ± 0.12	4.9 (3.9–5.8)
Kyphotic	Spherical	Pigmented	Short or absent	Long	C	before operculum at gill base	2.98 ± 0.47	7.1 (6.5–7.9)
Kyphotic	Spherical	Pigmented	?	?	?	?		
Kyphotic	Spherical	Pigmented	Short or absent	Long	C	Before operculum at gill base	1.94 ± 0.08	7.1 (6.8–7.2)
Kyphotic	Spherical	Pigmented	?	Long	C	Before operculum at gill base	3.27	6.1 (5.5–6.9)
?	?	Pigmented	Short or absent	Long	C	Before operculum at gill base	3.28	5.1 (3.7–5.7)
Kyphotic	Spherical	Pigmented	Short or absent	Long	C	Before operculum at gill base	3.08 ± 0.47	5.1 (5.0–5.3)
Kyphotic	Spherical	Pigmented	?	Long	C	Before operculum at gill base	1.76 ± 0.02	7.0 (5.8–7.8)
?	?	Unpigmented	Short or absent	?	Absent	?		
?	?	Unpigmented	?	?	Absent	?		
?	?	Pigmented	?	?	?	?		
?	Spherical?	?	?	?	?	?		
Straight	Oblong	Pigmented	?	?	?	?		
?	?	?	Short or absent	?	?	?		
Straight	Oblong	Pigmented	Long	Long	B	After operculum fused	4.50 ± 0.44	8.77 (8.5–9.2)
Straight	Oblong	Pigmented	Long	Long	B	After operculum fused	2.81 ± 0.15	10.6 (10.3–10.9)
Straight	Oblong	Pigmented	Short or absent	Short	A	Before operculum fused	2.72 ± 0.25	5.78 (5.4–6)
Straight	Oblong	Pigmented	Long	Long	B	After operculum fused	4.05 ± 0.09	10.5 (10.3–10.7)
Straight	Oblong	Pigmented	Short or absent	Short	A	Before operculum fused	2.67 ± 0.20	6.6 (5.8–7.3)
Straight	Oblong	?	Short or absent	Short	A	?		
Straight	Oblong	Pigmented	Short or absent	Short	A	Before operculum fused	2.39 ± 0.08	9.4 (8.5–10.5)
Straight	Oblong	?	Short or absent	Short	B	?		
Straight	Oblong	Pigmented	Long	Long	B	After operculum fused	2.71 ± 0.44	9.9 (9.3–10.1)
Straight	Oblong	Pigmented	Long	Long	B	Before operculum at gill base	4.2 ± 0.33	10.7
Straight	Oblong	Pigmented	Long	Long	B	After operculum fused	3.50 ± 0.18	10.9 (10.6–11.2)
Straight	Oblong	Pigmented	?	?	B	After operculum fused		
Straight	Oblong	Pigmented	?	?	B	?		
Kyphotic	Spherical	Unpigmented	Long	?	C	Before operculum at gill base		
Straight	Oblong	?	?	?	C	Before operculum at gill base		
Straight	Spherical	Pigmented	Short or absent	Long	D	?		
Kyphotic	Spherical	Unpigmented	Short or absent	Long	Absent	?		
?	Spherical	Pigmented	Short or absent	?	Absent	?		
Straight	Spherical	Pigmented	Short or absent	?	Absent	?		
Kyphotic	Spherical	Pigmented	Short or absent	?	Absent	?		
Kyphotic	Spherical	Unpigmented	Short or absent	?	Absent	?		
?	Spherical	Unpigmented	Short or absent	?	Absent	?		
Kyphotic	Spherical	Pigmented	Short or absent	?	Absent	?		

Appendix
Extended.

Adult SVL (mm)	Reference
	Gomez-Mestre et al. (2006)
	Mebs (1980)
	This work
	Gawor et al. (2012)
31.25	Salazar-Valenzuela (2007); this work
	Grillitsch et al. (1972)
	Rao and Yang (1994)
	Boulenger (1897); Richardson et al. (1997); Pennati et al. (2000); Nokhbatolfoghahai and Downie (2008)
	Iwasawa and Saito (1989)
	Iwasawa and Saito (1989)
48–120	Boulenger (1897); Dujsevayeva et al. (2004); Nokhbatolfoghahai and Downie (2008); Bekhet et al. (2014); this work
22 (20–24)	Izecksohn (1993); this work
	de Carvalho (1949); Izecksohn and da Cruz (1972)
	Izecksohn and da Cruz (1972)
	Khan (1965)
	Boulenger (1897)
	Limbaugh and Volpe (1957)
23.2 (19.4–27.8)	Baldo et al. (2014); Bornschein et al. (2015); this work
22.4 (18.3–25. 7)	Baldo et al. (2014); Bornschein et al. (2015); this work
23.3 (21.5–32.8)	Baldo et al. (2014); Bornschein et al. (2015); this work
	Kurth et al. (2014)
20.5 (18.0–24.4)	Baldo et al. (2014); Bornschein et al. (2015); this work
32.3 (27.7–37.8)	Baldo et al. (2014); Bornschein et al. (2015); this work
19.25 (17.6–23.7)	Baldo et al. (2014); Bornschein et al. (2015); this work
35.7 (32–42.7)	Baldo et al. (2014); Bornschein et al. (2015); this work
20.5 (19–24.9)	Baldo et al. (2014); Bornschein et al. (2015); this work
	Orton (1949); Lamotte and Xavier (1972b)
	Lamotte and Xavier (1972a)
	Dinesh and Radhakrishnan (2013); Chan et al. (2016)
	Inger (1960b)
	Díaz et al. (2000)
	Channing and Vences (1999)
61.5 (54–69)	Cei (1980); this work
100 (88–112)	Cei (1980); this work
43.8 (34.7–58.2)	Blotto et al. (2014); Narvaes and Trefaut Rodrigues (2009); this work
131.6 (110–178)	This work
54.7 (38.2–76.4)	Lavilla et al. (2000); Narvaes and Trefaut (2009); this work
	Nokhbatolfoghahai and Downie (2008)
54.1 (35.8–81.1)	Narvaes and Trefaut (2009); this work
	Nokhbatolfoghahai and Downie (2008)
71.3 (54.5–74.9)	Baldissera et al. (2004); this work
50.4 (42.2–57)	Carrizo (1992); Haad et al. (2014); this work
65–92	Vellard (1959); this work
	Ba-Omar et al. (2004)
	Sedra and Michael (1961); Balinski (1969); Barbault (1984)
	Nokhbatolfoghahai and Downie 2005
	León-Ochoa and Donoso-Barros (1974)
	Nokhbatolfoghahai and Downie (2005)
	Nokhbatolfoghahai and Downie (2005)
	Hervas et al. (2015)
	del Pino et al. (2004)
	Hervas et al. (2015)
	Salazar-Nicholls and del Pino (2015)
	Nokhbatolfoghahai et al. (2015)
	Salazar-Nicholls and del Pino (2015)