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Larval development and metamorphosis of the olfactory and vomeronasal organs in the toad *Rhinella* (*Bufo*) *arenarum* (Hensel, 1867)

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Abstract

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The olfactory and the vomeronasal system are the two major chemosensory systems found in terrestrial vertebrates. Among tetrapods, amphibians are unique in having an aquatic larval stage, followed by metamorphosis to a terrestrial adult. In the present work, we studied the histological development of the olfactory and vomeronasal organ and associated multicellular glands of the toad *Rhinella* (*Bufo*) *arenarum*, from early poshatching larva to postmetamorphic toadlets. As in other bufonids, the olfactory epithelium of *R. arenarum* in larvae is divided into dorsal and ventral branches in the rostral and mid-nasal regions. At metamorphic climax, the larval pattern changes drastically and the adult olfactory configuration develops. Bowman's glands appear in the olfactory epithelium of *R. arenarum* at the onset of metamorphic climax. The vomeronasal epithelium develops early in larval development in *R. arenarum*, around the time of operculum development. Interestingly, a novel sensory epithelium develops in the floor of the principal chamber of *R. arenarum* at metamorphic climax. This novel sensory epithelium resembles larval sensory epithelium lacking Bowman's glands, and suggests that these animals would be able to sense not only air-borne, but also water-borne odors during their adult terrestrial life.

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Introduction

Chemodetection of molecules in the external environment is essential for an organism's survival and reproduction (Prasad and Reed 1999). All vertebrates possess an olfactory organ as the major chemosensory system. Moreover, terrestrial vertebrates (tetrapods) have an accessory chemosensory system, the vomeronasal system, which is not present in teleost fishes and has been secondarily lost in crocodilians, birds, most bats, marine mammals and Old World primates (Bertmar 1981; Bhatnagar and Meisami 1998; Halpern and Martinez-Marcos 2003). Because the presence of a 'dual' nasal chemosensory system, with anatomically discernible olfactory and vomeronasal organs (VNOs), represents a synapomorphy for tetrapods, Bertmar (1981) hypothesized that vomeronasal system originated as an adaptation to terrestrial life. Nevertheless, a clear vomeronasal system has been found in fully aquatic salamanders belonging to Amphiumidae and Sirenidae families (Eisthen 2000). Moreover, the vomeronasal system is present in amphibians throughout life and does not arise at the metamorphic climax, as one might expect if the feature was an adaptation to terrestriality (Eisthen 1997). For that reason, Eisthen (2000) rejected Bertmar's hypothesis and concluded that the vomeronasal system arose in aquatic tetrapods, rather than as an adaptation to terrestrial life.

Among tetrapods, amphibians are unique presenting an aquatic larval period, followed by metamorphosis to a terrestrial juvenile form. This change in lifestyle dramatically affects the anatomy, physiology and ecology of amphibians, mainly

of anurans (Duellman and Trueb 1986). The olfactory and vomeronasal system of amphibians have been studied in caecilians (Badenhorst 1978; Billo and Wake 1987), caudates (Dawley and Bass 1988; Eisthen *et al.* 1994; Dawley and Crowder 1995; Eisthen 1997, 2000; Steulpnagel and Reiss 2005), and anurans (Born 1876; Hinsberg 1901; Watanabe 1936; Rowedder 1937; Cooper 1943; Tsui 1946; Yvroud 1966; Northcutt and Royce 1974; Scalia 1976; Khalil 1978a,b; Taniguchi *et al.* 1996a,b; Reiss and Burd 1997; Hansen *et al.* 1998; Jermakowicz *et al.* 2004; Wang *et al.* 2008). The VNO appears early during development in most anuran amphibians, around the time of operculum development, and long before hindlimb development (Cooper 1943; Nieuwkoop and Faber 1956; Taniguchi *et al.* 1996a,b; Tsui 1946; Wang *et al.* 2008). Exceptions occur in members of the family Bufonidae, in which the VNO is absent during the first half of the larval period and develops during its second half (Khalil 1978a,b; Jermakowicz *et al.* 2004). Interestingly, the olfactory epithelium (OE) arrangement in bufonids also differs from other neobatrachian amphibians during development. In fact, throughout larval period, the rostral region of the OE of bufonids is divided into two segments, dorsal (DOE) and ventral olfactory epithelium (VOE) (Khalil 1978a,b; Jermakowicz *et al.* 2004); while in other neobatrachian frogs the rostral OE does not divide into two distinct branches (Cooper 1943; Scalia 1976; Taniguchi *et al.* 1996a,b; Tsui 1946; Wang *et al.* 2008; Zwilling 1940). Given that branching of the rostral OE has only been reported in bufonids, Jermakowicz *et al.* (2004) proposed that this morphological character could represent a synapomorphy for bufonid tadpoles.

Clearly, variability in the nasal region of anuran amphians exists. This variation in shape and developmental timing of olfactory and VNOs could reflect different ecological challenges or phylogenetic history (or both) in anurans.

In the present work, we describe the ontogeny of the olfactory and VNOs, and associated multicellular glands, of the toad *Rhinella* (*Bufo*) *arenarum*, from posthatching periods to postmetamorphic stages, using conventional histology, lectin-histochemistry and immunohistochemistry for neural cell adhesion molecule (NCAM), which has been previously used for identification of olfactory neurons during development of *R. arenarum* (Paz *et al.* 1995).

Materials and Methods

Animals

Rhinella (*Bufo*) *arenarum* embryos were obtained by *in vitro* fertilization according to Casco *et al.* (1992). Tadpoles were maintained in dechlorinated tap water, with constant photoperiod and temperature (12 h : 12 h, dark : light; 22 ± 2 °C), and fed *ad libitum* with boiled chard. All animals were staged according to Gosner (1960). Newly metamorphosing toadlets were fed with live crickets and maintained in moist and humid terraria during 3 months. All experiments were performed in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA Res CD: 140/00, and the principles of the NIH (publication 8523, revised 1996).

Histological procedures

A total of 100 animals, ranging from G23 to G46 and 3-month-old juveniles (four animals each), were used in this study. After anesthetized by immersion in 0.1% MS222 (tricaine methanesulfonate; Sigma, St. Louis, MI, USA), animals were decapitated, and heads fixed in Bouin's solution for 24 h at 4 °C. Then, they were dehydrated in graded concentrations of ethanol, cleared in xylene and embedded in Histoplast (Biopack, Buenos Aires, Argentina). Serial transverse sections were cut at 5 μm (for G23–25 animals) or 7 μm (for G26–46–Juveniles animals) and mounted on HiFix glass slides (HF-5001; InProt, TNT, Buenos Aires, Argentina). For histological analysis, sections were deparaffined in xylene, rehydrated through a series of graded alcohols, stained with cresyl violet and mounted for conventional light microscopy.

Immuno- and lectin histochemistry

Tissue sections, obtained as described above, were deparaffined, rehydrated and washed in PBS. Non-specific binding sites were blocked by treating tissues with TNB blocking reagent (Cat. FP1020; NEN Life Science Products, Boston, MA, USA). Sections were incubated with the primary antibody, mouse anti-NCAM (Dr U. Rutishauser, Developmental Studies Hybridoma Bank, University of Iowa) 1/50, for 24 h at 4 °C. Then, samples were treated with the appropriate biotinylated antibody (Vector Laboratories, Burlingame, CA, USA) followed by streptavidin–Alexa fluor 488 complex (1/200; Molecular Probes). Specificity of the immunostaining was determined by omission of the primary antibody. The control sections produced negligible background staining. To enhance visualization of multicellular glands, lectin histochemistry was performed using a set of lectins which has showed to be specific to identify the associated nasal glands (Carmanchahi *et al.* 2000). Briefly, after deparaffining and rehydration, tissue sections were blocked for non-specific binding as described above, and incubated with the biotin-labeled lectins (Vector Labs) RCA (*Ricinus communis Agglutinin*) or WGA (*Triticum vulgaris Agglutinin*) overnight at 4 °C, and then treated with streptavidin–Alexa fluor 488 complex (1/200; Molecular Probes). All sections were counterstained with propidium iodide and coverslipped with glycerin-PBS (50/50, v/v). Images were captured by a confocal laser microscope (Olympus FV-30 attached to a microscope Olympus Bx-61).

Results

Late hatchling period

G23–25 . The olfactory organs are embedded in connective tissue lateral to the rostral end of the cornua trabeculae. At G23, the external nares open on the anterodorsolateral surface of the head. The principal chamber (PC) is already formed and the OE extends forward to the rostral end of the olfactory organ, closely associated with the external nares; it is located dorsolateral to the cornua trabeculae (Fig. 1A). The VNO has not developed yet. In the mid-region, the OE is divided into two branches, the DOE (dorsolaterally situated to the cornua trabeculae) and the VOE (ventrolaterally situated to the cornua trabeculae) (Fig. 1B). These two branches of the OE (DOE and VOE) correspond to the 'upper sac' (*Hauptlumen* for Rowedder 1937; and *Sac Supérieur* for Yvroud 1966) and to the 'posterior lower sac' (*Choanengang* for Rowedder 1937; and *Sac Choanal* for Yvroud 1966) of other authors, respectively (Rowedder 1937; Yvroud 1966).

The PC of the olfactory organ connects somewhat caudally with the buccal cavity. At G24–25, a small outpocketing of vomeronasal epithelium appears in the anteroventral portion of the DOE (Fig. 1C). At this time, the vomeronasal glands (VNG) have not developed.

Larval period

G26–30 . The PC of the olfactory organ is ovoid and extends along the longitudinal axis of the chondrocranium. It is lined with sensory epithelium medially and with non-sensory, non-ciliated epithelium laterally (Fig. 2B–D). The VNO is represented by a bean-shaped outpocketing anteroventral to the PC. At the rostral end of the nasal region, the VNO is situated dorsolateral to the cornua trabeculae, and only the ventral portion of the OE is present ventrolateral to the cornua trabeculae (Fig. 2A). The external nares are located at the rostral end of the PC, and are surrounded by a flap of tissue that forms a valvelike structure. At the midrostral region of the olfactory organ, the sensory epithelium is divided into two branches, the DOE and the VOE. The VOE has its apical surface exposed to the buccal cavity (Fig. 2B). In the mid-nasal region, a portion of non-sensory epithelium, which was not immunoreactive for the neuronal marker NCAM, separates the DOE and the VOE (Figs 2C and 7A). The external nares, the PC, and the choana form a direct dorsoventral channel from the external environment to the buccal cavity. This feature is maintained in all stages of larval development. Caudally, the DOE and the VOE merge together to form one continuous epithelium. The DOE has shifted slightly dorsally to the roof of PC and forms a horseshoe-shaped structure at the most caudal region. As with the external nares, a flap of tissue hangs off the medial and lateral walls of the choana, forming a valvelike structure. Caudally, the medial valve flap becomes longer (Fig. 2D).

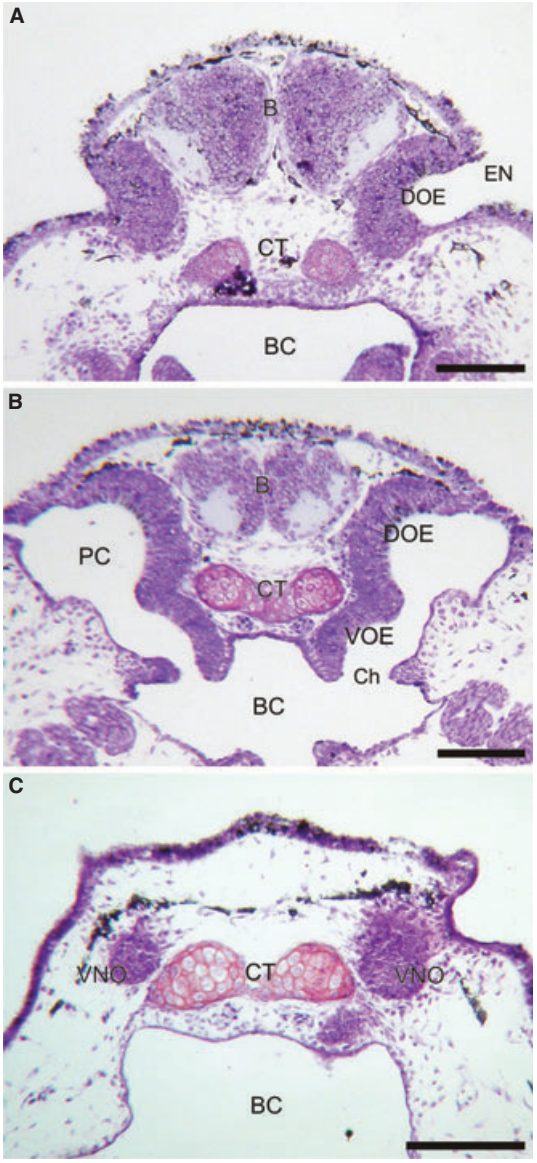

Fig. 1—Transverse sections of *Rhinella arenarum* at late hatchlings period. —**A**,**B.** Animals at G23 stages at rostral and mid-regions of the nasal cavity, respectively. Note that the OE is divided into two branches at the mid-nasal region, the DOE (**A**) and the VOE (**B**). —**C.** At G25, the VNO appears as a small outpocket at the rostral nasal region, dorsolateral situated to the CT. Bars: 100 μm. B, brain; BC, buccal cavity; Ch, choana; CT, cornua trabeculae; DOE, dorsal olfactory epithelium; EN, external nares; PC, principal chamber; VNO, vomeronasal organ; VOE, ventral olfactory epithelium.

By G28–29, the VNG develop in the connective tissue medial to the VNO, and dorsal to the cornua trabeculae. They develop rostrally to the DOE and dorsally to the VOE (Fig. 6A).

G31–39 . As the larva develops, the VNO continues growing and expanding medially forming a medial diverticulum at the

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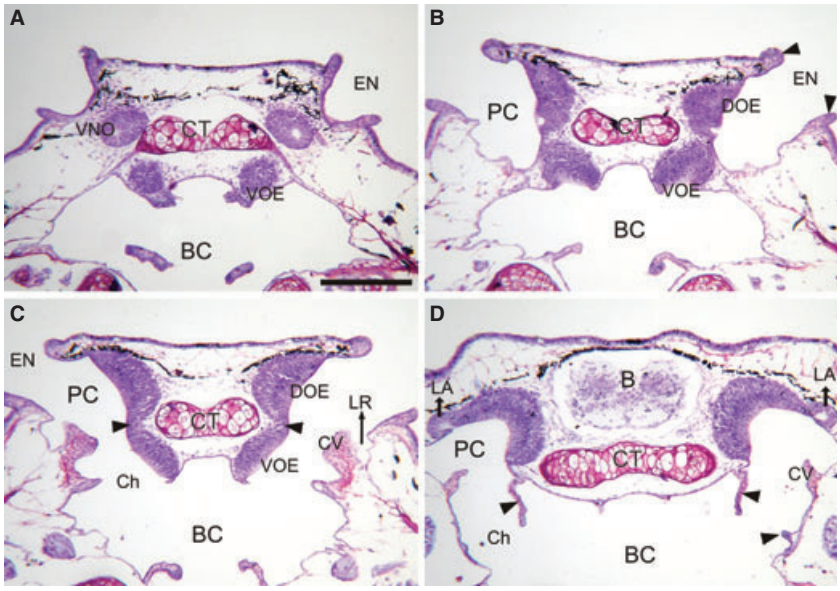

Fig. 2—Transverse sections of G28 tadpoles at rostral (**A**), midrostral (**B**), middle (**C**), and caudal regions (**D**) of the nasal cavity. —**A.** At the rostral nasal end, the VNO and the VOE appears dorsolateral and ventrolateral to the CT respectively. —**B.** At the midrostral region, the DOE appears in the middle wall of the PC whereas the VOE is exposed to the BC. The PC opens to the external environment via the EN which is surrounded by a valve-like structure (Arrowheads). —**C.** At the mid-nasal region, both the DOE and the VOE line the medial wall of the PC. A portion of non-sensory epithelium separates the DOE and the VOE (Arrowheads). The EN, the PC, and the Ch form a direct dorsoventral channel from the external environment to the BC. —**D.** At the caudal region, the PC is contiguous with the buccal cavity via the Ch. A flap of tissue hangs off the medial and lateral walls of the PC forming a valve-like structure (Arrowheads). Bar: 200 μm. B, brain; BC, buccal cavity; Ch, choana; CT, cornua trabeculae; CV, crista ventralis; DOE, dorsal olfactory epithelium; EN, external nares; LA, lateral appendix; LR, lateral Rinne; PC, principal chamber; VNO, vomeronasal organ; VOE, ventral olfactory epithelium.

mid-rostral end of the PC (Fig. 3A). Typical bipolar neurons, immunoreactive for NCAM, are observed in the VNO (Fig. 7B). The VNG grow in the connective tissue around the VNO, forming evident branched tubular glands (Figs 3A,B and 6B). Despite the increase in size of the nasal region, its general morphological characteristics remain essentially the same (Fig. 3). Rostromedially, the VOE remains exposed to the buccal cavity. At the mid-region of the olfactory organ, the DOE and the VOE continue to be separated (Fig. 3B), and both sensory epithelia merge together caudally (Fig. 3C). The DOE becomes thigher at the mid-caudal region, showing bipolar olfactory neurons extending its apical process to the epithelial surface (Fig. 7C). By G35–36, the cartilaginous nasal septum begins to develop between the right and left OE in the caudal region, and the DOE becomes encapsulated by the developing olfactory capsule at G37–38 (Fig. 3D).

G40–41 . The DOE begins to expand rostrally and the VNO continues to increase in size, causing the DOE, the VOE, and the VNO to be in the same dorsoventral plane. Development of the olfactory capsule continues to encapsulate the DOE, and separate it from the VNO (Fig. 4A). By G41, the middle chamber (MC) develops at the rostral end of the nasal region, dorsally situated to the VNO, and anteroventral to the PC. The choana remain ventrolateral to the DOE, and start to migrate caudally. At G41, the VOE lines the dorsal aspect of the buccal cavity, similar to early stages, but in a more caudal position. A primordial tongue begins to develop at the bottom of the buccal cavity. In the DOE (but not in the VOE) mucous vesicles from developing Bowman's glands are visible (Fig. 6C). The PC increases in size and the surface area of the OE increases as well. At the mid-caudal region of the olfactory organ, the OE expands to cover not only the medial surfaces, but also the ventral and dorsal lining of the PC (Fig. 4B).

Metamorphic climax

G42–45 . The external nares shift ventrolaterally and are located on the lateral side of the head at G43. The DOE continues expanding rostrally, which causes it to be the first sensory epithelium that appears at the rostral nasal end (Fig. 4C). A novel sensory epithelium, immunoreactive for the neuronal marker NCAM, develops in the floor of the rostral region of the PC during metamorphic climax (G43–44) (Figs 4C and 7D). The PC and the dorsoventrally flattened MC increase in size. The nasolacrimal ducts develop and connect laterally with the MC. Nasal cartilages are well developed and subdivide the nasal region. The inferior chamber (IC) develops ventrally to the middle of the PC. It is dorsoventrally

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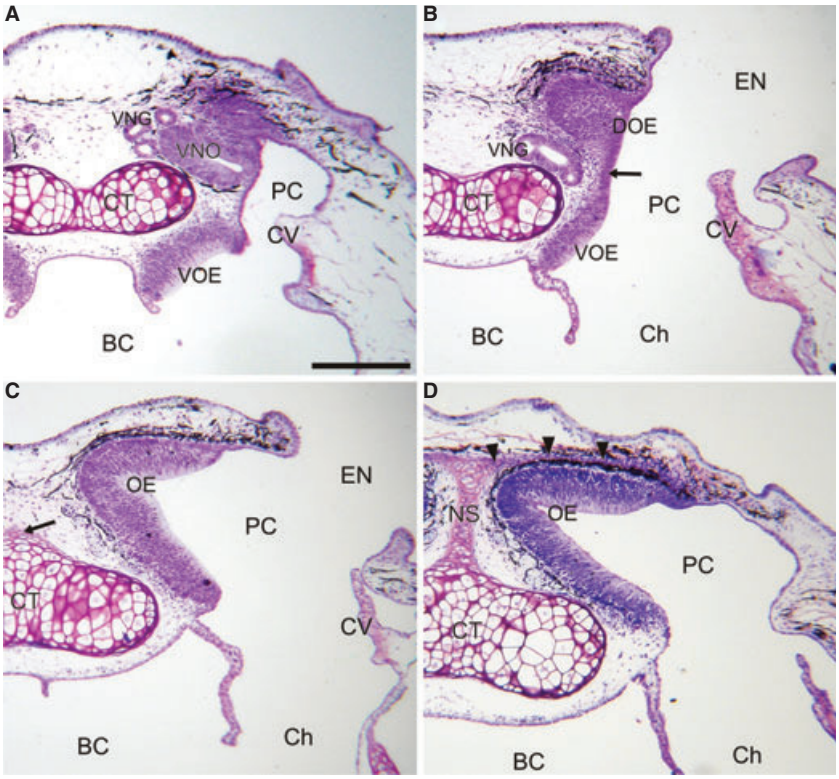

Fig. 3—Transverse sections (right side) at rostral (**A**), mid- (**B**), and caudal (**C**) nasal regions of G35 tadpoles, and caudal nasal region of G38 tadpoles (**D**). —**A.** Rostrally, the VNO appears dorsolateral to the CT. Tubular VNG proliferate in the connective tissue surrounding the VNO. The VOE is exposed to the BC. —**B.** At the mid-region, the DOE is located close to the EN whereas the VOE is exposed to the ventral aspect of the BC. The DOE and the VOE are separated by a portion of non-sensory epithelium (Arrow). —**C.** Caudally, the DOE and the VOE merge together forming a continuous epithelium at the medial wall of the PC. The nasal septum begins to develop between left and right PC (Arrow). —**D.** The cartilaginous olfactory capsule begins to develop encapsulating the OE (Arrowheads). Bar: 200 μm. BC, buccal cavity; Ch, choana; CT, cornua trabeculae; CV, crista ventralis; DOE, dorsal olfactory epithelium; EN, external nares; NS, nasal septum; OE, olfactory epithelium; PC, principal chamber; VNG, vomeronasal glands; VNO, vomeronasal organ; VOE, ventral olfactory epithelium.

flattened, with a medial diverticulum lined with a growing vomeronasal epithelium, and a lateral diverticulum lined with ciliated non-sensory epithelium (Fig. 4D). The PC, the MC, and the IC are interconnected with each other. At the mid-nasal region, the DOE occurs on the lateral, dorsal, and medial surface of the PC. Caudally, the PC is lined with sensory epithelium on the medial, dorsal, and dorsolateral surfaces, and connects ventrally with the buccal cavity. The VOE shifts further caudally and disappear by G43–44 (Fig. 4E). During metamorphic climax, the larval mouth parts are lost and the tongue and associated structures develop.

G46–juveniles . Metamorphosis concludes and the head and associated nasal region resemble the adult phenotype. The external nares are located at the lateral side of the head, opening into the ventral section of the PC, at its rostral end. The OE is enclosed in the olfactory capsule, and lines the medial wall and the roof of the PC at the rostral nasal end. A concave vestibule develops in the floor of the PC between the OE and the external nares (Fig. 5A). This 'canal like' structure connects caudally with the MC and the IC. The novel sensory epithelium, developed in the floor of the PC during metamorphic climax, increases in size. This sensory epithelium is separated from the OE by connective tissue and mucus glands, which correspond to the 'glandula oralis interna' of Helling (1938) (Fig. 5B). Bowman's glands are not present in this sensory epithelium, while they proliferate and become clearly noticeable within the metamorphosed OE (Figs 5 and 6D). The MC is entirely lined with non-sensory epithelium and is located at the rostral portion of the nasal region, ventral to the PC (Fig. 5C). The IC extends mediolaterally perpendicular to the PC at the mid-portion of the nasal region. It is lined with non-sensory epithelium in the lateral diverticulum whereas the medial diverticulum is lined with the sensory epithelium of the VNO, which has remarkably increased in size (Figs 5C,D and 7E). In the middle regions of the PC, the OE occurs on all surfaces, except where the PC connects to the MC or IC.

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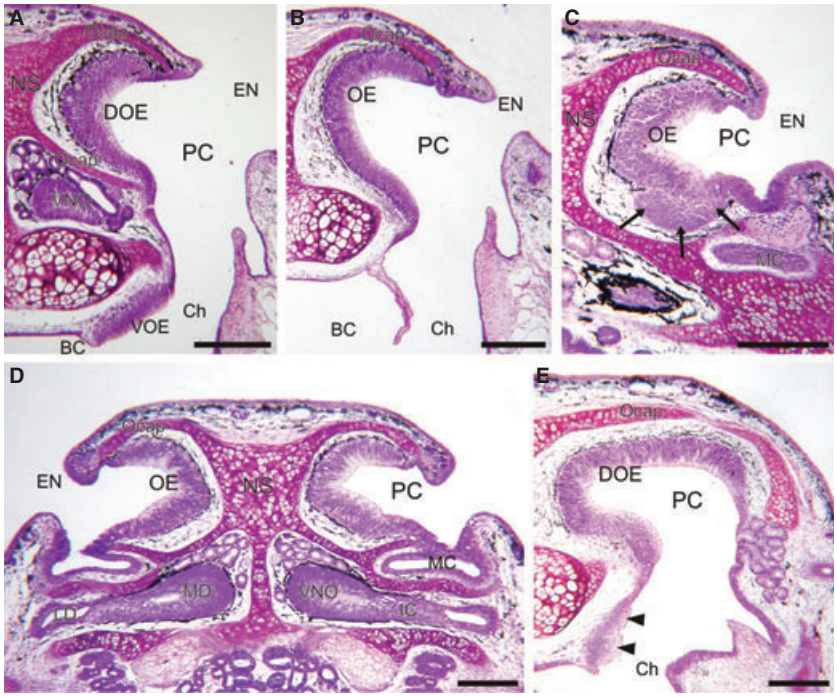

Fig. 4—Transverse sections of late larval stage animals (G41; **A**,**B**), and metamorphosing animals (G43, **C–E**). —**A.** The DOE expands rostrally, and the VNO increases in size causing the DOE, the VNO, and the VOE to appear in the same dorsoventral plane. —**B.** Caudally, the OE lines the medial wall and the roof of the PC. —**C.** A novel sensory epithelium develops in the floor of the rostral PC, close to the EN (Arrows). —**D.** Nasal cartilages are well developed and subdivide the three nasal chambers: the PC, the developing MC, and the IC, which develops a medial diverticulum (MD) lined with a growing VNO and a lateral diverticulum (LD) lined with ciliated non-sensory epithelium. —**E.** Caudally, the DOE lines the dorsomedial wall and the roof of the PC, which connects with the buccal cavity via the Ch. A portion of degenerating VOE can be seen in the ventromedial wall of the PC, close to the Ch (Arrowheads). Bars: 200 μm. BC, buccal cavity; Ch, choana; DOE, dorsal olfactory epithelium; EN, external nares; IC, inferior chamber; LD, lateral diverticulum; MC, middle chamber; MD, medial diverticulum; NS, nasal septum; OCap, olfactory capsule; OE, olfactory epithelium; PC, principal chamber; VNO, vomeronasal organ; VOE, ventral olfactory epithelium.

At the most caudal nasal region, the PC becomes dorsoventrally flattened. The choana open at the lateroventral side of the PC, connecting it with the buccal cavity. The OE occurs on all surfaces of the olfactory organ. The floor of the PC elevates forming the olfactory eminence, which is covered by a thick OE (Fig. 5E).

Discussion

The larval OE of *Rhinella* (*Bufo*) *arenarum* is divided into dorsal and ventral parts in the rostral nasal end. This particular feature has only been reported in bufonids (Jermakowicz *et al.* 2004; Khalil 1978a,b; Rowedder 1937). Therefore, the hypothesis that branching of the rostral OE may represent a synapomorphy for bufonids tadpoles remains as a possibility. Nevertheless, analysis of other members of the Bufonidae family are necessary to confirm this hypothesis.

During larval stages, the olfactory organ is highly specialized in association with the small mouth opening. The DOE occurs on the dorsomedial wall of the PC, near the external nares, whereas the VOE exists in the dorsal portion of the buccal cavity (rostrally) or the ventromedial wall of the PC (more caudally). Probably, both the DOE and the VOE detect water-borne odorants flowing throughout the PC via the external nares, but the rostral portion of the VOE may also serve as a chemosensory detector in the buccal cavity. This particular disposition of the sensory epithelia might enhance chemosensory sampling of environmental cues during the larval period. Through metamorphic climax, the VOE shifts caudally and disappears, and the tongue and associated structures develop. So, it is possible that the VOE (exposed to the buccal cavity) may contribute to chemosensory detection in buccal cavity of tadpoles as do taste buds in the adult tongue.

The VNO is represented by a bean-shaped outpocketing which develops from anteromedial portion of the OE in anurans (Cooper 1943; Khalil 1978a,b; Rowedder 1937; Scalia 1976; Taniguchi *et al.* 1996a,b; Tsui 1946; Wang *et al.* 2008; Zwilling 1940). Development of VNO typically occurs earlier in most anuran amphibians. In the mesobatrachian *Xenopus laevis*, the VNO appears at Nieuwkoop and Faber (N/F) stage 37/38, before operculum development (N/F 40) (Nieuwkoop

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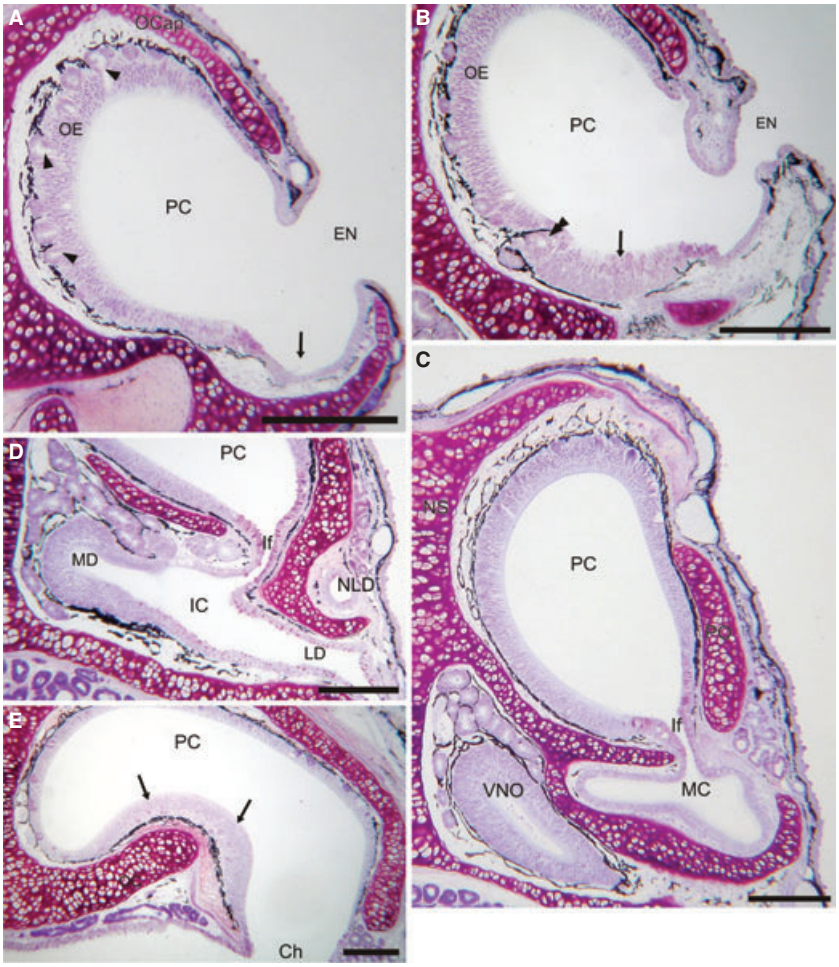

Fig. 5—Transverse sections of 3-month-old juvenile animals (right side). —**A.** The EN opens at the lateral side of the anterior PC (ventrally situated). The OE appears somewhat elevated, lining mainly the medial wall and the roof of the PC. Bowman's glands are clearly noticeable (Arrowheads). A concave vestibule develops in the floor of the PC (Arrow). —**B.** The sensory epithelium, developed in the floor of the PC during metamorphosis, increases in size (Arrow); it is separate from the OE by connective tissue and mucus glands (Double-Arrowhead). Note the absence of Bowman's glands in this sensory epithelium. —**C.** At the mid-region, the OE lines all aspect of the PC except where it connects with the non-sensory MC. The VNO has remarkably increased in size. —**D.** In a more caudal region than (C), the MC disappears and the PC connects ventrally with the IC, which is lined with the VNO at the medial diverticulum (MD) and non-sensory epithelium at the lateral diverticulum (LD). —**E.** Caudally, the PC became flattened, connecting ventrolaterally with the BC. In the floor of the PC a thick OE elevates forming the olfactory eminence (Arrows). Bars: 200 μm. Ch, choana; EN, external nares; IC, inferior chamber; If, infundibulum; MC, middle chamber; NLD, nasolacrimal duct; OCap, olfactory capsule; OE, olfactory epithelium; PC, principal chamber; VNO, vomeronasal organ.

and Faber 1956). In the neobatrachians *Rana pipiens*, *Rana japonica*, *Rana nigromaculata*, and *Rana chensinensis* the VNO develops early, around the time of operculum development (G21–23) (Cooper 1943; Taniguchi *et al.* 1996a,b; Tsui 1946; Wang *et al.* 2008). Even in the directly developing frog *Eleutherodactylus coqui*, which bypasses the aquatic tadpole stage and hatches directly as a terrestrial anuran in the adult phenotype, the VNO develops early in embryonic periods (Jermakowicz *et al.* 2004). Exceptions occur in the bufonids *Bufo americanus* and *Bufo regularis*, in which the VNO is absent during the first half of the larval period and develops during its second half (G34 and G30–32, respectively; Jermakowicz *et al.* 2004; Khalil 1978a). Here, we show that in the bufonid *R. arenarum*, the VNO appears early in development, around the time of operculum development (G24–25), like in most anuran amphibians. Moreover, VNG appear slightly later than VNO in *R. arenarum* (G28–29). In amniotes and most anurans, VNG development trails VNO ontogeny (Holtzman and Halpern 1990; Taniguchi *et al.* 1996b; Garrosa *et al.* 1998; Wang *et al.* 2008), whereas, in the bufonids *B. regularis* and *B. americanus* VNG emerge at the same time as the VNO.

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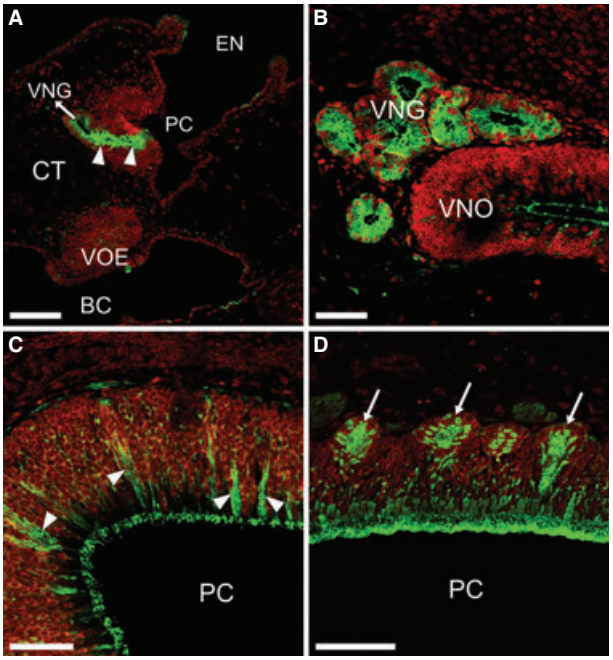

Fig. 6—Lectin histochemistry staining of VNG (using *Ricinus communis Agglutinin*, RCA lectin) (**A**,**B**), and Bowman's glands staining (using *Triticum vulgaris*, WGA lectin) (**C**,**D**). —**A.** At G29, the VNG develops in the connective tissue medial to the VNO**.** They discharge their secretion products onto the lateral portion of the VNO, close to the connection between the VNO and the medial wall of the PC (Arrowheads). —**B.** VNG proliferate throughout development forming branching tubular glands in the connective tissue around VNO (G39). —**C.** At late larval period (G41), mucus droplets from developing Bowman's glands are visible in the DOE (Arrowheads). —**D.** At juvenile stages, Bowman's glands are abundant in the OE (Arrows). They slightly protrude into the connective tissue under the OE. Bars: 100 μm (A), 50 μm (B–D). BC, buccal cavity; CT, cornua trabeculae; EN, external nares; PC, principal chamber; VNG, vomeronasal glands; VNO, vomeronasal organ; VOE, ventral olfactory epithelium.

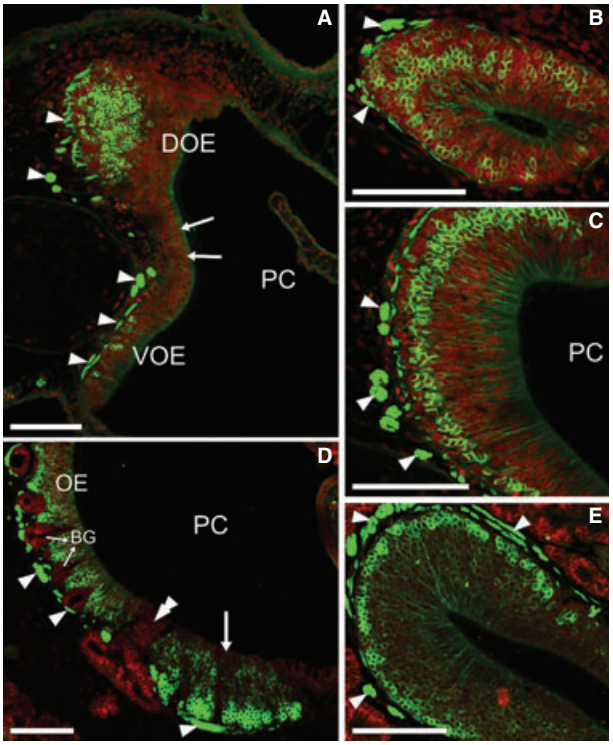

Fig. 7—Immunohistochemistry for neuronal marker NCAM at different developmental stages. —**A.** At G29, the DOE and the VOE are separated by a portion of non-sensory epithelium, which is not immunoreactive for NCAM (Arrows). —**B.** Transverse section of the right VNO of G37 tadpoles, showing the sensory neuron distribution. —**C.** NCAM immunostains sensory neurons in the DOE at G37. —**D.** At juvenile stages, the novel sensory epithelium developed in the floor of the anterior PC during metamorphic climax is intensely immunostaining for NCAM (Arrow), mucus glands (Double-Arrowhead) separate it from the metamorphosed OE. Bowman's glands are abundant in the OE but not in the novel sensory epithelium. —**E.** Transverse section of the right VNO of a 3-month-old juvenile. Arrowheads show axon bundles at the *lamina propria* in all panels. Bars: 100 μm. BG, Bowman's glands; DOE, dorsal olfactory epithelium; OE, olfactory epithelium; PC, principal chamber; VOE, ventral olfactory epithelium.

The major known function of the VNO is to detect chemical signals arising from conspecifics (Halpern and Martinez-Marcos 2003). Tadpoles utilize chemical cues in several social communications like kin recognition (Blaustein and Waldman 1992), social foraging (Sontag *et al.* 2006), and alarm pheromone detection (Fraker *et al.* 2009). Despite the early appearance of VNO in tadpoles' development, there is no evidence about functional or maturational state of these sensory epithelia during aquatic larval periods. It would be interesting to explore whether the VNO serves to detect social chemicals in tadpoles.

Bowman's glands begin to develop in the PC of *R. arenarum* during metamorphic climax (G41–43), and are clearly noticeable before the end of metamorphosis (G45). In all anurans studied to date, Bowman's glands appear in the OE of the PC only during metamorphic climax, and are completely lacking in the premetamorphic (larval) PC (Hansen *et al.* 1998; Jermakowicz *et al.* 2004; Khalil 1978a,b; Taniguchi *et al.* 1996b; Wang *et al.* 2008). Hansen *et al.* (1998) found Bowman's glands in the PC (air sensitive) of postmetamorphic *Xenopus laevis*, whereas they were absent in the postmetamorphic MC (water sensitive) or larval PC. Thus, in anurans, the appearance of Bowman's glands in the PC seems to be a metamorphic event. These data suggest that they may not be necessary for stimulus-sensing in aquatic environments. Moreover, the increase in number and maturity of Bowman's glands in postmetamorphic animals suggest that increased mucus secretion occurs in terrestrial forms. This is likely necessary not only to protect the epithelium against desiccation,

but also to secrete odorant-binding proteins to aid in transporting volatile molecules across the layer of mucus.

During metamorphic climax, the larval nasal region changes drastically and the adult olfactory configurations develops. Cartilaginous structures delimitate the three interconnected chambers: the PC, the newly formed MC, and the IC, with its lateral and medial (vomeronasal) diverticulum.

Interestingly, a novel sensory epithelium develops in the floor of the anterior region of the PC during metamorphic climax in *R. arenarum*. This sensory epithelium resembles the larval OE lacking associated Bowman's glands. Anatomical disposition and histological features suggest that this sensory epithelium would enable *R. arenarum* to continue smelling in water after metamorphosis. We have recently described the presence of segregated populations of olfactory receptor neurons (coupled to different transduction pathways) in the larval OE of *R. arenarum* (Jungblut *et al.* 2009). It would be exciting to explore whether different populations of olfactory receptor neurons are present in the adult larval-type sensory epithelium described here. Interestingly, a larval-type sensory epithelium has been described in the terrestrial coastal giant salamander *Dicamptodon tenebrosus* (Steulpnagel and Reiss 2005), and in the aquatic adult of the caecilian *Typhlonectes compressicauda* (Saint Girons and Zylberberg 1992). Surprisingly, somewhat resembling the situation in *R. arenarum*, the larval-type sensory epithelium of these amphibians is anteroventrally situated in the nasal region, and lacks associated Bowman's glands. By examining older studies on anuran olfactory system, we found that Helling (1938) described a special region in the OE of adult anurans close to the external naris, the *recessus olfactorius*, which lacks Bowman's glands and might be involved in aquatic olfaction. It seems that the *recessus olfactorius* is not equally developed in the different families of anuran amphibians. For instance, it is completely lacking in Hylidae, Ranidae, Rhacophoridae and Microhylidae; whereas, it is poorly developed in the family Bombinatoridae. On the other hand, an evident developed *recessus olfactorius* is present in Pelobatidae, Cycloramphidae, Ceratophryidae, Leptodactylidae and particularly Bufonidae, in which it seems to be clearly developed (Helling 1938). It would be exciting to investigate whether the presence or absence of larval-type sensory epithelium is a function of habitat characteristics or phylogenetic history (or both).

Adults of the secondarily aquatic frog *Xenopus laevis* have sensory epithelium lining both the PC and the MC. The MC is always filled with water and serves the animal to sense water-borne odorants, whereas the PC is filled with air and detects air-borne odorants (Altner 1962). The presence of sensory epithelium in the MC appears to be unique to *Xenopus* and other pipids (Paterson 1951) and is clearly functionally correlated with the aquatic lifestyle of adults. Most anurans have only a main OE, located in the PC, and a VNO (Scalia 1976). Adults of *R. arenarum*, like other bufonids, are generally not aquatic; although, like most amphibians, they do return to water to breed. Therefore, it could be possible that, given the absence of sensory epithelium lining the MC, this larval-type sensory epithelium developed in the floor of the PC serves the animals to detect environmental molecules when they return to water to breed. Further studies using experimental approaches and morphology-based functional interpretations are necessary to confirm this hypothesis.

Social chemical communication has been well described in salamanders (Dawley 1998; Houck 2009). Nevertheless, bioacoustic signals seem to be so essential to the social communication of anuran amphibians that other sensory modalities have been largely ignored. However, since the description of the tree frog *Litoria splendida* aquatic sex pheromone 'splendipherin' a decade ago (Wabnitz *et al.* 1999), anuran chemical communication has become a flourishing area of physiological investigations, and a growing body of evidence suggests that it would be essential in social interactions (Belanger and Corkum 2009; Houck 2009).

Nevertheless, data concerning anuran chemical communication are still very scarce. Moreover, despite the earlier description of the presence of a larval-type sensory epithelium in anuran amphibians, there are no studies that explore which sensory epithelium (vomeronasal, main OE, or larval-type OE) is activated when animals respond to aquatic chemical stimuli.

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